NOTE TO USERS

This reproduction is the best copy available.

UMI®

UNIVERSITY OF ALBERTA

Vaccinia Virus Encodes a Novel Mitochondrial Inhibitor of Apoptosis that Interacts with Bak and Inhibits Bak Activation

©

By

Shawn Todd Wasilenko

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree Doctor of Philosophy
in
Virology

Department of Medical Microbiology and Immunology
Edmonton, Alberta
Fall 2005

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

0-494-08751-X

Your file Votre référence
ISBN:
Our file Notre reference
ISBN:

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

Apoptosis is a cellular death process that operates in all metazoans. In response to virus infection, apoptosis can be initiated by cellular stress, cell surface receptors, and by direct attack of cytotoxic T lymphocytes and natural killer cells. Activation of apoptosis results in the proteolytic activation of caspases, a family of cysteine proteases, that mediate death of the cell. In most cases, apoptosis proceeds through mitochondria which undergo a number of hallmark changes including loss of the inner mitochondrial membrane potential and release of pro-apoptotic proteins such as cytochrome c. Bcl-2 proteins tightly regulate apoptosis at mitochondria. Following an apoptotic stimulus, pro-apoptotic Bcl-2 proteins Bak and Bax are activated and modulate the release of cytochrome c which is prevented by anti-apoptotic Bcl-2 family members.

Many viruses encode proteins that block distinct steps of apoptotic cascades. These include caspase inhibitors such as the poxvirus-encoded caspase 8 inhibitor, CrmA/Spi-2, and mitochondrial localized virus-encoded Bcl-2 homologs. Since the majority of apoptotic cascades proceed through mitochondria, we hypothesized that vaccinia virus utilized a mechanism to regulate the mitochondrial apoptotic pathway. We found that infection with a CrmA/Spi-2 deficient strain of vaccinia virus prevented apoptosis by regulating the mitochondrial checkpoint. Since vaccinia virus does not encode proteins with homology to mitochondrial inhibitors of apoptosis, we utilized a panel of deletion viruses to identify F1L as a novel inhibitor of apoptosis. F1L is a tail-anchored protein that is exclusive to Orthopoxviruses (Stewart *et al.* 2005). F1L localizes to mitochondria and functions to inhibit loss of the inner mitochondrial membrane potential and release of cytochrome c. F1L constitutively associates with Bak

and functions to prevent Bak activation following the induction of apoptosis. During our studies, we also found that F1L expression was necessary to prevent apoptosis induced by vaccinia virus infection. In contrast to wildtype vaccinia virus, Jurkat cells infected with two F1L deletion viruses, VV811 and VV(Cop)ΔF1L, triggered loss of the inner mitochondrial membrane potential and release of cytochrome c independent of prior caspase activation. Overall, our data indicates F1L is a novel mitochondrial-associated inhibitor of apoptosis that interferes with Bak activation and is necessary for the inhibition of vaccinia virus-induced apoptosis.

ACKNOWLEDGEMENTS

Simply put, the last six years have been challenging. In some respects, this thesis is a reminder of good and bad times over this time frame but more than anything, this thesis is a collection of ideas and progress that each of you have achieved with me. At this time I give thanks for I can finally say, WE did it!! I love you all and am truly blessed for each of you in my life. For my wife, Lotje, whom I love more with every breath I take and every beat of my heart. You are my pillar of strength, my ray of sunshine and my rainbow of joy. Mom and Dad, for your constant love, support, optimism and funding. You are both what I strive to become as an adult and a parent. Natasha, we share a unique and strong bond that is as special as your loving family Blair, Tatiana and Connor. Thank-you all for your support, optimism and kicking me in the backside when I needed it. Grandma Nadya, for your love and support, keep the good dreams coming. Henk and Marjo, my new family, for allowing me to bring Lotje to Canada and loving me as a son. God, for blessing me with re-assurance, strength, guidance and His comforting hand. Thank-you for my support network and all the goodness, including the trials, You have blessed me with. This is truly for Your glory.

TABLE OF CONTENTS

Chapter 1: General Introduction 1

1.1 Overview of Poxviruses 2

1.1.1 Poxvirus Diversity and Pathogenesis 2

1.1.2 The Poxvirus Virion 4

1.1.3 The Poxvirus Life Cycle 6

1.1.4 Orthopoxviridae 9

1.1.5 Vaccinia Virus 9

1.1.6 Immune Modulation by Poxviruses 10

1.2 Classification of Cell Death 10

1.2.1 Apoptosis 12

1.2.2 Autophagy 14

1.2.3 Necrosis 15

1.3 Caspases 16

1.3.1 Mechanism of Caspase Activation 17

1.3.2 Negative Regulation of Caspases 21

1.4 Apoptotic Pathways 22

1.4.1 The Intrinsic Apoptosis Pathway 23

1.4.2 The Extrinsic Apoptotic Pathway 23

1.4.3 The Granzyme B Apoptosis Pathway 27

1.5 Mitochondria and Apoptosis 29

1.5.1 Mitochondrial Structure and Function 29

1.5.2 Mitochondrial Permeabilization during Apoptosis 32

1.5.3 Bcl-2 Family Members Regulate Apoptosis at the Mitochondria 33

1.5.3.1 Structural properties of Bcl-2 proteins 35

1.5.3.2 BH3–only proteins: sentinels of apoptotic signals 37

1.5.3.3 Multidomain proapoptotic Bax and Bak 40

1.5.3.4 Anti-apoptotic Bcl-2 proteins 43

1.5.4 Mechanisms of Cytochrome c Release 44

1.5.4.1 The mitochondrial permeability transition model 45

1.5.4.2 The outer membrane pore model 47

1.5.4.3 The mitochondrial remodelling model 48

1.6 Viral Anti-apoptotic Strategies 50

1.6.1 Inhibition of Death Receptor Mediated Apoptosis 52

1.6.2 Interference of DISC Formation and Function 54

1.6.3 Viral Encoded Caspase Inhibitors 55

1.6.4 Viral IAPs 57

1.6.5 Virus-encoded Proteins that Regulate the Mitochondria 58

1.6.5.1 Viral Bcl-2-like proteins 58

1.6.5.2 Viral non-Bcl-2 like proteins 61

1.7 Thesis Objectives 64

1.8 References 66

Chapter 2: Vaccinia Virus Infection Disarms the Mitochondrion-Mediated Pathway of the Apoptotic Cascade by Modulating the Permeability Transition Pore 84

2.1 Introduction 85

2.2 Materials and Methods 87

Cell and Viruses. 87

Virus Infection.. 88

Antibodies. 88

Apoptosis Induction. 89

Chromium Release Assay.. 90

Cytochrome c Release Assay... 90

Measurement of Mitochondrial Membrane Potential.................................. 90

Detection of DNA Fragmentation.. 91

In vitro Reconstitution Assay... 92

Immunoblotting. 93

2.3 Results 93

Vaccinia Virus strain Copenhagen-infected Cells are Resistant to anti-Fas-mediated Apoptosis. 93

Vaccinia Virus strain Copenhagen Infection Inhibits Cytochrome c Release in Isolated Mitochondria but not Cleavage of Bid. 97

Vaccinia Virus strain Copenhagen-infected Cells are Resistant to Staurosporine-mediated Apoptosis. 101

Vaccinia Virus Infection Inhibits Disruption of the Mitochondrial Inner Membrane Potential and Opening of the PTP. 105

2.4 Discussion 111

2.5 References..118

Chapter 3: Vaccinia Virus Encodes a Novel Mitochondrial Associated Inhibitor of Apoptosis..122

3.1 Introduction..123

3.2 Materials and Methods..126

Cells and Viruses..126

Virus Infection..126

Plasmid Construction. 127

Generation of Stable Transfected Cell Lines. 129

Apoptosis Induction..129

Detection of DNA Fragmentation..130

Cellular Fractionation. 130

Infection and Transfection Assay..131

Transient Transfections. 131

Measurement of Mitochondrial Membrane Potential..132

Confocal Microscopy. 132

Immunoblotting. 134

3.3 Results..135

Vaccinia Virus Infection Disrupts the Mitochondrion Component of the Apoptotic Cascade. 135

Vaccinia Virus Deletion Mutants have Distinguishable Affects on Apoptosis 137

F1L Protects VV811 Infected Cells from Staurosporine- and Fas-induced Loss of the Inner Mitochondrial Membrane Potential. 143

The Vaccinia Virus F1L Protein Localizes to Mitochondria. 147

The C-terminal Hydrophobic Domain of F1L is Critical for Mitochondrial Localization. 149

F1L Expression is Sufficient to Block Loss of the Inner Mitochondrion Membrane Potential. 154

F1L Inhibits Apoptosis by Blocking Release of Cytochrome c and SMAC. 157

F1L Expression Protects Cells from VV811-induced Apoptosis. 161

3.4 Discussion 163

3.5 References 169

Chapter 4: The Vaccinia Virus F1L Protein Interacts with the Proapoptotic Protein Bak and Inhibits Bak Activation 173

4.1 Introduction 174

4.2 Materials and Methods 177

Cells and Viruses 177

Generation of F1L Deletion Virus. 177

Gel Filtration Analysis 178

Immunoaffinity Purification. 179

Immunoprecipitation. 179

Mitochondria Purification and Cytochrome c Release. 180

Confocal Microsccopy. 181

Bak Conformational Analysis by Flow Cytometry. 182

Measurement of Mitochondrial Membrane Potential. 182

Immunoblotting. 183

4.3 Results 183

F1L Expression Protects Cells from Apoptosis. 183

Vaccinia Virus F1L Protein Specifically Interacts with Bak. 189

F1L Inhibits Staurosporine-induced Bak Activation. 193

F1L Regulates tBid-induced Bak Activation. 197

4.4 Discussion 201

4.5 References 207

Chapter 5: Discussion 210

5.1 Summary of Thesis 211

5.2 The Anti-apoptotic Mechanism of F1L 216

5.3 Vaccinia Virus Induces Apoptosis 225

5.4 Future Directions 230

5.5 References 232

Appendix – Supporting Methods and Results 237

A-1 Stable Expression of F1L Inhibits Staurosporine-induced Loss of the Inner Mitochondrial Membrane Potential....238

A.2 Transcription Profile of F1L Stable Clones....239

- A.2.1 Method for RT-PCR....239
- A.2.2 Results....239

A.3 Stable Expression of F1L Protects Cells from Granzyme B-induced Apoptosis....241

- A.3.1 Method for the Induction of Granzyme B-induced Apoptosis....241
- A.3.2 Results....241

A.4 VV811 Induces Apoptosis in Infected Cells Directed Through the Mitochondrial Cascade....245

- A.4.1 Method for the Induction of VV811-induced Apoptosis....245
- A.4.2 Results....246

A.5 F1L Expression Inhibits Staurosporine-induced Bak Activation....248

- A.5.1 Method for Monitoring the N-terminal Exposure of Bak....251
- A.5.2 Results....251

A.6 References....253

LIST OF TABLES AND FIGURES

CHAPTER 1

Table 1-1 The poxvirus family 3

Figure 1-1 Structure of poxvirus virions 5

Figure 1-2 The poxvirus lifecycle 7

Table 1-2 Poxvirus encoded anti-apoptotic proteins 11

Table 1-3 Morphological, biochemical and pathological comparison of apoptosis, necrosis and autophagy 13

Figure 1-3 Phylogenic analysis, substrate specificity and function of mammalian caspases 18

Figure 1-4 Mechanisms of caspase activation 19

Figure 1-5 Schematic representation of the intrinsic apoptotic cascade 24

Figure 1-6 Schematic representation of the Fas receptor-mediated extrinsic apoptotic cascade 26

Figure 1-7 Schematic representation of the granzyme B-mediated apoptotic cascade 28

Figure 1-8 Mitochondrial structure and pro-apoptotic proteins released during apoptosis. 31

Figure 1-9 The Bcl-2 family 34

Figure 1-10 Ribbon representation of Bcl-2 family members 36

Figure 1-11 Structural similarities of Bcl-XL and diphtheria toxin 38

Figure 1-12 Classical models of cytochrome c release Figure 1-3 Phylog 46

Figure 1-13 Mitochondrial cristae remodeling and cytochrome c release..................49

Figure 1-14 Viral inhibitors of apoptosis...51

CHAPTER 2

Figure 2-1 Vaccinia virus strain Copenhagen protects cells from anti-Fas-mediated death 95

Figure 2-2 Vaccinia virus strain Copenhagen infection inhibits activation of caspase 3 96

Figure 2-3 Vaccinia virus strain Copenhagen inhibits cytochrome c translocation..98

Figure 2-4 Vaccinia virus infection protects against granzyme B-mediated cytochrome c release from isolated mitochondria by a mechanism downstream of Bid activation...100

Figure 2-5 DNA fragmentation is blocked by vaccinia virus strain Copenhagen infection 103

Figure 2-6 Staurosporine-induced caspase 3 activation is inhibited by vaccinia virus strain Copenhagen infection 104

Figure 2-7 Staurosporine-induced cytochrome c release is inhibited by vaccinia virus strain Copenhagen infection...106

Figure 2-8 Vaccinia virus strain Copenhagen infection inhibits staurosporine-induced disruption of the mitochondrial membrane potential 108

Figure 2-9 Vaccinia virus strain Copenhagen inhibits opening of the PTP 110

Figure 2-10 Model of vaccinia virus-mediated apoptosis inhibition 116

CHAPTER 3

Figure 3-1 Vaccinia virus strain Copenhagen protects cells from staurosporine-induced apoptosis 136

Figure 3-2 Vaccinia virus deletion mutant VV811 is unable to protect cells from staurosporine-induced apoptosis. 139

Figure 3-3 Vaccinia virus deletion mutant VV811 is unable to protect cells from staurosporine-induced disruption of the inner mitochondrion membrane potential 142

Figure 3-4 The vaccinia virus F1L open reading frame restores the ability of VV811 to maintain the inner mitochondrion membrane potential 146

Figure 3-5 The product of the vaccinia virus F1L open reading frame localizes to mitochondria 148

Figure 3-6 F1L is highly conserved within the Orthopoxvirus genus 151

Figure 3-7 The C-terminal hydrophobic domain of F1L is necessary and sufficient for mitochondria localization...153

Figure 3-8 F1L expression in the absence of virus infection maintains the inner mitochondrial membrane potential following apoptosis 156

Figure 3-9 F1L inhibits cytochrome c and SMAC release from mitochondria 160

Figure 3-10 VV811 induces apoptosis that is inhibited by F1L 162

CHAPTER 4

Figure 4-1 Characterization of a recombinant VV devoid of F1L 184

Figure 4-2 VV(Cop)ΔF1L is unable to protect cells from staurosporine-induced apoptosis 186

Figure 4-3 VV(Cop)ΔF1L induces apoptosis in Jurkat cells......................................188

Figure 4-4 F1L interacts with endogenous Bak but not other Bcl-2 family proteins191

Figure 4-5 F1L interacts with Bak in the presence and absence of VV(Cop) infection192

Figure 4-6 F1L and endogenous Bak localize at the mitochondria during vaccinia virus infection 194

Figure 4-7 F1L expression inhibits staurosporine-induced Bak activation..............196

Figure 4-8 F1L inhibits tBid-induced Bak activation...199

APPENDIX A

Table A-1 Stable expression of F1L is sufficient to block the loss of the inner mitochondrial membrane potential.. 238

Figure A-1 Transcript profile of Jurkat cells stably expressing F1L........................240

Figure A-2 Stable expression of F1L prevents granzyme B-induced DNA fragmentation 242

Figure A-3 Stable expression of F1L prevents granzyme B-induced loss of the inner mitochondrial membrane potential....244

Figure A-4 VV811 induces the loss of the inner mitochondrial membrane potential247

Figure A-5 VV811 infection induces the hallmark features of mitochodrial dependent apoptosis.... 250

Figure A-6 Stable expression of F1L prevents staurosporine-induced N-terminal exposure of Bak....252

LIST OF ABBREVIATIONS

AD	adenovirus
AIF	apoptosis inducing factor
AmEPV	*Amsacta moorei* entomopoxvirus
ANT	adenine nucleotide translocator
Apaf-1	apoptotic protease activating factor-1
Apo2L/TRAIL	TNF-related apoptosis inducing ligand
ASFV	African swine fever virus
ATP	adenosine tri-phosphate
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X-protein
BCA	bicinchoninic acid
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-XL	B-cell leukemia/lymphoma protein Xl
BH	Bcl-2 homology domain
Bid	BH-3 interacting domain death agonist
BIR	baculovirus inhibitor of apoptosis repeat
CAD	caspase activated DNAase
CAML	calcium-modulating cyclophilin ligand
CARD	caspase-recruitment domain
ClCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
CrmA	cytokine response modulator A
CTL	cytotoxic T lymphocyte

DD	death domain
DED	death effector domain
Diablo	direct IAP-binding protein with low PI
DISC	death inducing signaling complex
DNA	deoxyribonucleic acid
DR4	death receptor 4
DR5	death receptor 5
dsRNA	double stranded RNA
EEV	extracellular enveloped virion
EGFP	enhanced green fluorescent protein
EndoG	Endonuclease G
ER	endoplasmic reticulum
EV	ectromelia virus
FADD	Fas-associated death domain
$FADH_2$	reduced flavin adenine dinucleotide
FasL	Fas ligand
FBS	Fetal bovine serum
FLICE	Fas-associated death-domain-like interleukin-1β converting enzyme
FLIPS	Fas-associated death domain like interleukin-1β converting enzyme inhibiting protein
GB	granzyme B
HtrA2	high temperature required protein A

HCMV	human cytomegalovirus
HPV	human papillomavirus
HVEM	high-voltage electron microscopy
IBM	IAP binding motif
IAP	inhibitor of apoptosis protein
ICAD	inhibitor of caspase activated DNAase
ICE	interleukin-1β-converting enzyme also known as caspase-1
IMV	intracellular mature virion
KS-Bcl-2	Kaposi's sarcoma herpesvirus Bcl-2
MCMV	murine cytomegalovirus
MCV	molluscum contagiosum virus
MOI	multiplicity of infection
MOPS	morpholinepropanesulfonic acid
NADH	reduced nicotinamide adenine dinucleotide
NF-κB	nuclear factor kappa-B
NK	natural killer
NP-40	nonionic detergent P-40
ORF	open reading frame
Op-IAP	*orgyia pseudotsugata*
PBR	peripheral benzodiazepine receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLIC1	protein-linking integrin-associated protein and cytoskeleton 1

PFU	plaque forming units
PT	permeability transition
PTP	permeability transition pore
RID	receptor internalization and degradation
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Serp-2	Serine protease-2
SMAC	Second mitochondria-derived activator of caspase
Spi-2	Serine protease inhibitor-2
STS	staurosporine
TMRE	tetramethylrhodamine ethyl ester
TNFα	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TRADD	TNF receptor associated death domain
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling
vBCL-2	viral Bcl-2
VDAC	voltage dependent anion channel
vFLIP	viral FLIP
vIAP	viral IAP
vMIA	viral mitochondrial inhibitor of apoptosis
VV	vaccinia virus

XIAP	X-linked inhibitor of apoptosis

Chapter 1: General Introduction

1.1 Overview of Poxviruses

Poxviruses comprise a large family of DNA viruses. The most notorious member of the family is variola virus, the causative agent of smallpox. The first cases of smallpox are thought to have occured nearly 3000 years ago (91). More recently, smallpox was responsible for claiming the lives of nearly 500 million people and scarring millions in the 20th century alone (91, 253). The high mortality and morbidity associated with smallpox infection as well as the availability of a safe vaccine led the World Health Organization to pursue the global eradication of smallpox (91). With a budget of twenty-four million dollars and a vaccination program that emphasized surveillance and containment, smallpox was globally eradicated in 1977 (91). Despite the eradication of smallpox, new global problems involving variola virus and other poxviruses persist. The increased frequency of zoonotic poxvirus infections and the threat of variola re-emerging as a biological weapon are just two issues that serve to remind the scientific community that understanding these pathogens is critical (13, 172).

1.1.1 Poxvirus Diversity and Pathogenesis

The Poxviridae family is categorized into two subfamilies based on viral host cell tropism (216). *Chordopoxvirinae* are vertebrate-tropic viruses and *Entomopoxvirinae* are insect-tropic viruses (Table 1-1) (216). The *Chordopoxvirinae* are further divided into eight genera including *Orthopoxviridae, Leporipoxviridae, Avipoxviridae, Capripoxviridae, Suipoxviridae, Molluscipoxviridae, Yatapoxviridae, and Parapoxviridae* (Table 1-1) (216). The *Entomopoxvirinae* are divided into three genera and are designated *Entomopoxviridae A, B,* and *C* (Table 1-1) (216).

Family Poxviridae

Subfamily	Genus	Viruses
Chordopoxvirinae **(vertebrate)**	*Orthopoxviridae*	Buffalopox‡, camelpox, cowpox‡, ectromelia, monkeypox‡, raccoonpox, skunkpox, taterapox, uasin gishu, vaccinia‡, variola‡, volepox
	Leporipoxviridae	Hare fibroma, myxoma, rabbit fibroma, shope fibroma, squirrel fibroma
	Avipoxviridae	Canarypox, fowlpox, juncopox, mynahpox, pigeonpox, psittacinepox, peacockpox, penguinpox, quailpox, sparrowpox, starlingpox, turkeypox
	Capripoxviridae	Goatpox, lumpy skin disease, sheeppox
	Suipoxviridae	Swinepox
	Molluscipoxviridae	Molluscum contagiosum‡
	Yatapoxviridae	Tanapox‡, yaba-like disease‡, yaba monkey tumor
	Parapoxviridae	Auzuk disease, bovine papular stomatitis‡, chamois contagious ecthyma, orf‡, pseudocowpox, parapox of deer, paravaccinia‡, sealpox‡
Entmopoxvirinae (invertebrate)	*Entomopoxviridae A*	Melolontha melolontha
	Entomopoxviridae B	Amsacta moorei
	Entomopoxviridae C	Chironomus luridus

Table 1-1 The poxvirus family.
(‡) Denotes viral species reported to cause disease in humans.
Adapted from Moss, B 1996 (216).

Members of the Poxviridae induce acute infections in many animal species however, the host range and lethality of individual poxviruses are highly variable. Whereas some poxviruses such as vaccinia virus infect a wide range of hosts, others such as variola virus exhibit a narrow host range (90). Some poxviruses such as variola virus are highly lethal mainly due to systemic infection as a result of viral dissemination (90). Others, such as vaccinia virus, induce localized benign skin lesions which are cleared by the immune system in immunocompetent individuals (90).

1.1.2 The Poxvirus Virion

Poxvirus virions range in size from 270nm to 350nm (Figure 1-1) (76, 220). Each virion consists of a phospholipid membrane envelope surrounding aggregates of heterogeneous material referred to as lateral bodies and a complex dumbbell-shaped core structure housing a large, linear double stranded DNA genome with inverted terminal repeats and closed hairpin loops at each terminus (17, 103, 303). At present, thirty-eight poxvirus genomes have been sequenced. Poxvirus genomes range in size from 130kbp for bovine papular stomatitis virus genome to 360kbp for canarypox virus (69, 329). Comparison between twenty-six poxvirus genomes has led to the identification of ninety genes that are conserved in all chordopoxviruses and forty-nine genes conserved in all poxviruses (118, 333). All conserved genes are located in a central genomic region spanning ~100kbp that contains genes necessary for transcription, replication and virion assembly (118, 333). Towards the termini, many of the genes are unique and non-essential for viral replication in cell culture and encode proteins involved in host range and viral virulence (204, 244).

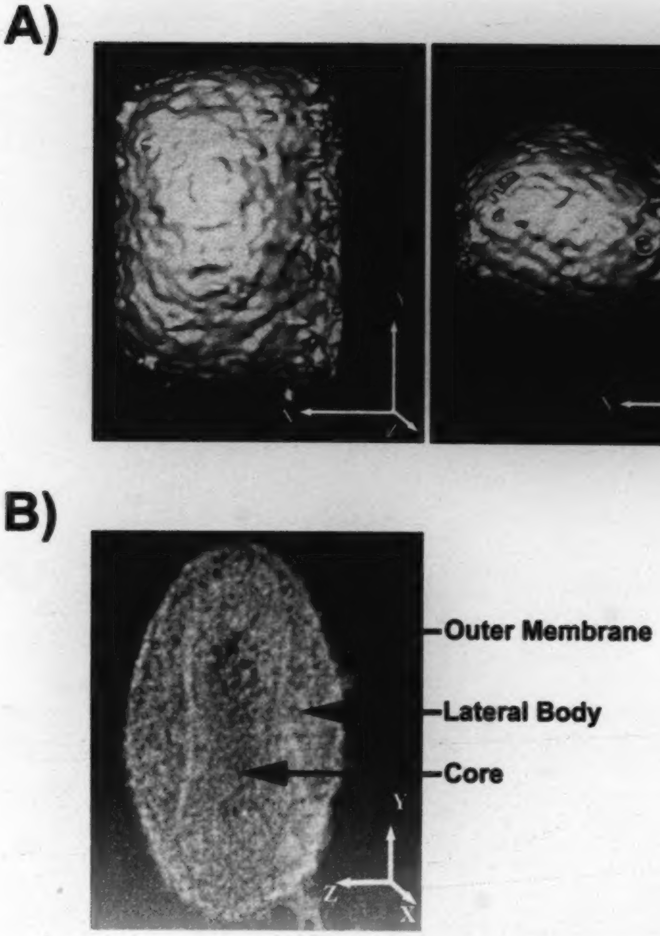

A)
B)
Outer Membrane
Lateral Body
Core
Y
Z
X

Figure 1-1 Structure of poxvirus virions.
(A) Surface rendering representation of vaccinia virus intracellular mature virion particle characterized by a barrel shaped, slightly compressed morphology. (B) Translucent visualization of thin sections of IMV particle revealing a complex internal structure. Adapted from Cyrklaff, M *et al.* 2005 (63).

1.1.3 The Poxvirus Life Cycle

In contrast to other DNA viruses, poxviruses replicate autonomously in the infected cell cytoplasm. The large genomes of poxviruses encode all the proteins necessary for their unique lifestyle (216). The poxvirus lifecycle is regulated by differential protein expression. Proteins expressed early are involved in viral DNA replication, intermediate gene transcription and immune evasion (37). Proteins expressed at the intermediate phase regulate late gene transcription and, along with proteins expressed late, are involved in virion morphogenesis and assembly (37).

The poxvirus lifecycle is structured into a number of distinct stages including the attachment and entry of the virion to a host cell, viral gene transcription, viral DNA replication, virion assembly and maturation followed by virus dissemination (Figure 1-2). The lifecycle of poxviruses is complex due in part to the production of different types of infectious virions. During replication intracellular mature virions (IMV), extracellular enveloped virions (EEV) and cell-associated enveloped virions (CEV) are produced and all possess the ability to infect host cells (29, 184, 337, 338). IMV particles are composed of the virion core and lateral bodies surrounded by an undefined number of lipid bilayers (Figure 1-1). While some studies suggest the lipid membrane is composed of a single bilayer, others suggests the lipid membrane is composed of two tightly opposed lipid bilayers (114, 133). During the replication cycle, IMV particles can obtain an additional lipid membrane to form EEV and CEV (241, 280).

Virus attachment and entry into a host cell are two poorly understood processes that are complicated by the fact that there are different types of infectious virions. CEV particles present on the cell surface are directly driven into surrounding cells by actin tails (302). EEV and IMV particles likely enter cells using different receptors which is

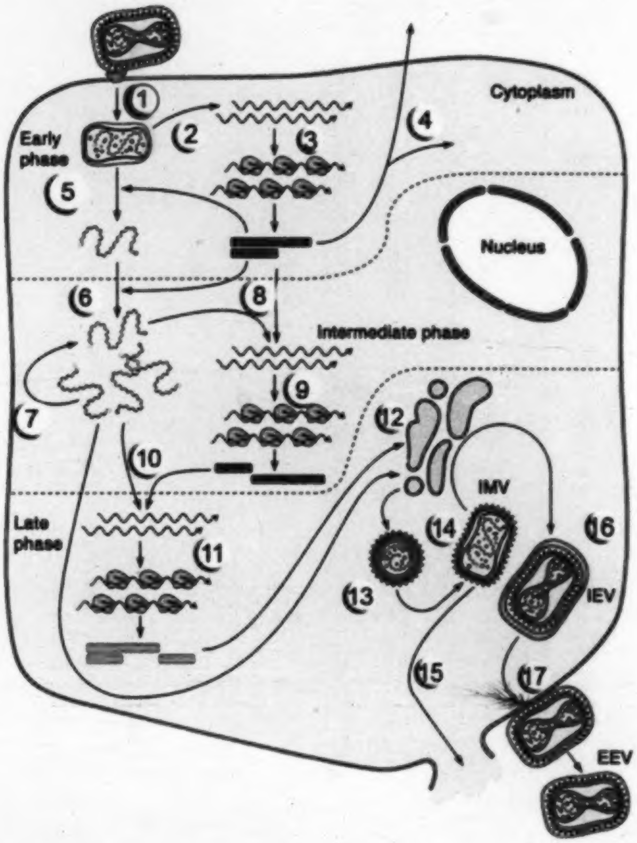


Figure 1-2 The poxvirus lifecycle.
Intracellular mature virion (IMV) or extracellular mature virion (EEV) bind to different ligands on the outer plasma membrane facilitating entry (1). Intra-core early gene transcription and uncoating stage 1 (2). Translation of early genes by host translational machinery (3). Newly translated early proteins are generated to counter host immune system defences (4), promote uncoating stage 2 to release viral DNA (5), and catalyze viral DNA replication (6). Newly synthesized viral DNA serves as templates for replication (7) and transcription of viral intermediate genes (8). Intermediate proteins are translated (9) and serve to promote late gene transcription (10) followed by late protein translation (11) and viral membrane protein synthesis (12). Virion assembly and production in which proteins and viral genomic material are wrapped by membrane crescents, of unknown origin, to generate an immature virion (IV) (13) that goes on to yield an intracellular mature virion (IMV) (14) which is released upon cell lysis (15). The IMV can be wrapped by additional lipid bilayer membranes from the trans-Golgi compartment to giving rise to an intracellular enveloped virion (IEV) (17). IEV move to the cell surface, fuse with the plasma membrane to form cell-associated virions (CEV) (17) that can dissociate from the membrane as extracellular enveloped virions (EEV). Adapted from Flint SJ *et al.* (2003) (97).

consistent with antigenically distinct surface proteins on each outer membrane (339). EEV and IMV particle entry into the cytoplasm requires the removal of all virion membranes. Since EEV are surrounded by an additional membrane, the entry mechanisms are likely to be different although this step in the replication cycle is poorly understood (338). Once entry is achieved however, all viral particles undergo a two-step uncoating process in which early viral mRNA is transcribed in the core and released (152, 216). The released transcripts are expressed and mediate further core uncoating that results in the release of the viral genome for replication (134, 147). Viral DNA replication and virion assembly take place in peri-nuclear regions termed viral factories which are rich in viral DNA and proteins (216). The first visible structures within the viral factory are crescent shaped lipid membranes containing viral proteins that expand and enclose viral DNA and proteins to form immature virions (IV) (65, 328). Maturation of IV involves condensation of the virus genomic material and proteolytic cleavage of several capsid proteins to yield IMV (217). The resulting IMV are fully infectious virions that represent the majority of infectious progeny that are released during lysis of the cell (216). Some IMV particles become enwrapped by two additional lipid membranes derived from the trans-Golgi network to form intracellular enveloped virions (IEV) (131, 280). IEV particles are transported to the cell surface and fuse with the plasma membrane to expose an enveloped virion (302). The enveloped virions are classified as CEV if retained on the cell membrane and EEV if released. CEV are important for cell-to-cell transmission while EEV mediate systemic spread of the virus (29, 240).

1.1.4 Orthopoxviridae

One could argue that the disciplines of virology, immunology and vaccinology originated from studies involving Orthopoxviruses. After all, variola virus was the first virus to be seen by microscopy, cowpox virus was the first agent used to prevent disease and smallpox was the first disease in which a preventative vaccine treatment was successfully used (90). Today, the *Orthopoxviridae* are by far the most intensively studied poxvirus genera likely due to their historical significance and the availability of excellent model systems to study viral pathogenesis (89, 90). Currently, fifteen Orthopoxvirus genomes, from six different species, have been completely sequenced. Phylogenic analysis indicates that members of this genera are closely related and were likely the last poxvirus genera to have diverged (118).

1.1.5 Vaccinia Virus

Vaccinia virus, a virus with greater than 90% homology to variola virus, is the prototypic member of the poxvirus family and has served to influence much of virology and vaccinology (204, 205). Vaccinia virus superseded cowpox virus as the vaccination tool of choice against variola virus infection in the mid-1800's (90). It was the first virus to be purified (298), grown in tissue culture (108), seen by electron microscope (267), and used to express foreign genes in animals (191, 235). Although no natural host for the virus has been identified, a recent theory proposes that the virus originated from horsepox virus (21, 240, 316). Whatever the natural host, vaccinia virus has proven to be a useful research agent in many scientific disciplines and continues to be the vaccine of choice against smallpox.

1.1.6 Immune Modulation by Poxviruses

In order to sustain a successful infection, all viruses have developed strategies to evade and manipulate the host immune system (325). Much of the interest in poxvirus biology has focused on genes that provide immune modulation. The fact that thirty-eight poxvirus genomes are sequenced has led to the identification of a large number of diverse protein families with potential immunomodulatory function. So diverse are these protein families that no single immunomodulatory gene is present in all poxviruses (286). Despite this diversity, all chordopoxviruses encode proteins that disrupt the interferon-inducible pathway, chemokine/cytokine pathways and the apoptotic pathway (20, 145, 286).

The ability to inhibit apoptosis is a recurring theme among viruses, and poxviruses have evolved unique strategies that target crucial steps in the apoptotic cascade. In some cases, poxvirus proteins that inhibit apoptosis share homology to known cellular proteins whereas others are unique. Poxvirus-encoded proteins that function to inhibit apoptosis are summarized in Table 1-2.

1.2 Classification of Cell Death

The last thirty years has seen a rapid advance in our understanding of the mechanism of cell death, as it was once thought to be a physiological process that occurred without any order (79, 185). Cell death is now understood to be a genetically controlled event under most conditions and is an important component of development, morphogenesis, tissue homeostasis and immune system function (62, 79, 171, 232, 293). Insufficient cell death clinically manifests as autoimmunity and cancer whereas

Protein	*Gene*	*Genus*	*Function*
TNF Receptor Decoys	**MT-2, CrmB, CrmC, CrmD, CrmE**	**Leporipoxvirus, Orthopoxvirus**	**Sequester extracellular TNF**
vFLIP	**MC-159**	**Molluscipoxvirus**	**DED interaction**
MV-LAP	**MYX-M153R**	**Capripoxvirus Leporipoxvirus Suipoxvirus**	**MHC Class I and CD4 Downregulation**
CrmA/Spi2	**VAC(WR)-B13R CPXV-B12R**	**Orthopoxvirus**	**Caspase-1 and -8 inhibitor**
Serp-2	**MYX-M151R**	**Capripoxvirus Leporipoxvirus Yatapoxvirus**	**Potential caspase/granzyme B inhibitor**
IAP	**AMV-021**	**Entomopoxvirus**	**Inhibits caspase activation**
vBcl-2	**FPV-039**	**Avipoxvirus**	**Predicted to function similar to cellular Bcl-2**
M11L	**MYX-M11L**	**Capripoxvirus Leporipoxvirus Suipoxvirus Yatapoxvirus**	**Interacts with PBR and Bak to inhibit mitochondrial-mediated apoptosis**
F1L	**VAC(Cop)-F1L**	**Orthopoxvirus**	**Inhibits mitochondrial-dependent apoptosis**
Bax-Inhibitor 1	**CMPV-036 CPXV-S1R MPXV-B1R**	**Orthopoxvirus**	**Unknown**
p28/N1R	**EVM-012 RFV-S153R**	**Avipoxvirus Capripoxvirus Leporipoxvirus Orthopoxvirus Suipoxvirus Yatapoxvirus**	**Inhibits UV-induced apoptosis**
Selenoprotein	**MC-066L FPV-064**	**Avipoxvirus Molluscipoxvirus**	**Modulates ROS production**
Pyrin Domain	**MYX-013L RFV-013L**	**Orthopoxvirus,**	**Domain predicted to fold similar to DD, DED, and CARD domains**

Table 1-2 Poxvirus encoded anti-apoptotic proteins. AMV, amsacta moorei virus; CMPV, camelpox virus; CPXV, cowpox virus; EVM, ectromelia virus; FPV, fowlpox virus; MC, molluscum contagiosum virus; MYX, myxoma virus; RFV, Rabbit fibroma virus; SFV, Shope fibroma virus; VAC, vaccinia virus. Adapted from Barry, M *et al.* 2005 (20).

accelerated cell death leads to chronic degenerative diseases and immunodeficiency (62, 112, 146, 171, 234, 293). As shown in Table 1-3, cell death can be distinguished as apoptosis, autophagy, and necrosis based on specific morphological and biochemical features (111, 160, 185).

1.2.1 Apoptosis

Apoptosis was initially described in the early 1970s as an "alternative cell death" phenomenon to necrosis and was hypothesized to be a genetically pre-ordained process that regulated cellular homeostasis (153). Following the morphological characterization of apoptosis, very little research was directed at understanding the mechanism for this type of cell death. The identification of caspases as critical mediators of apoptosis and the discovery of B-cell leukemia/lymphoma 2 (Bcl-2) as a novel oncogene that transformed cells by preventing death however, resulted in a robust interest in apoptosis (54, 340, 366-368). It is now recognized that apoptosis is a tightly regulated and irreversible cell death process that is functional in all metazoans. Apoptosis plays a critical role in development, maintenance of normal cellular homeostasis, regulation of the immune system and removal of dangerous cells (18, 146, 232, 234).

Apoptosis is an adenosine triphosphate (ATP)-driven cell-suicide program that results in the degradation of cellular proteins and organelles (53, 80, 168, 263). The morphological features associated with apoptosis include chromatin condensation, cytoplasmic condensation, DNA fragmentation and the formation of plasma membrane blebs called apoptotic bodies that are removed by phagocytic cells thereby avoiding activation of the inflammatory response (77, 153). Apoptosis is activated by diverse

		Apoptosis	*Autophagy*	*Necrosis*
Morphological Features	*Nucleus*	Chromatin condensation Nuclear fragmentation DNA laddering (~200bp fragments)	Partial chromatin condensation Nucleus intact until late stages No DNA laddering	No regulated nuclear degradation pattern
	Cytoplasm	Cytoplasmic condensation Fragmentation to apoptotic bodies Cytoskeleton breakdown	Increased number of autophagic vesicles Increased number of autolysosome number Enlarged Golgi and dilated ER Functional cytoskeleton	Osmotic swelling of the cytoplasm
	Plasma Membrane	Membrane Blebbing	Membrane Blebbing	Membrane Rupture
Biochemical Features	*Bioenergetic State*	Physiological levels of ATP	Maintenance of ATP	Loss of ATP
	Protease Activity	Caspase dependent	Caspase-independent Increase in lysosomal protease activity	Caspase-independent
Pathology	*Clearance*	Cleared by phagocytes	Self clearing Occasionally cleared by other cells at late times	Cell bursts into extracellular milieu
	Inflammation	No inflammation	No inflammation	Inflammation

Table 1-3 Morphological, biochemical and pathological comparison of apoptosis, necrosis and autophagy.
Adapted from Gozuacik, D. and Kimchi, A. 2004 (111); Kroemer, G *et al* 1998 (160); Lockshin, RA and Zakeri, Z 2004 (185).

stimuli and yet always terminates with the same morphologic features which are attributed to the activation of a caspase dependent biochemical pathway. The activation of caspases coordinates the dismantling of a cell's structural and regulatory components that disrupts the essential physiological functions of the cell (54, 226, 321). Caspase activation is a unique feature of apoptosis that distinguishes apoptosis from other types of cell death (79, 185).

1.2.2 Autophagy

Autophagy is a highly conserved degradation and recycling pathway that functions primarily to maintain cellular homeostasis and provide bioenergetic metabolites during periods of nutrient starvation and growth factor deprivation (212). Autophagy has been implicated in a number of diseases including neurodegenerative disorders, myopathies and cancer (62, 230). Induction of autophagy generates double membrane bound vesicles called autophagosomes that amass cytoplasmic contents including organelles and proteins destined for degradation (199). Lysosomal hydrolases acquired by the fusion of autophagosome to an autolysosomes are used to generate metabolites that will produce energy for the cell (199). Since autophagy is a cannibalistic process that results in atrophy of the cell, it has been proposed to be an alternative form of cell death. A number of studies have implicated autophagy in cell death induced by viral infection, endoplasmic reticulum (ER) stress, toxins, and chemotherapeutic drugs (64, 150, 233, 317). Thus the initial activation of autophagy is likely a cellular survival mechanism to generate ATP however, cells that pass a "salvage threshold", a point at which the cell has exhausted all its cellular metabolites, will undergo an autophagic cell death (188).

Cells undergoing autophagy are characterized by a number of morphological and biochemical features (Table 1-3) (111). The appearance of autophagic vesicles remains the gold standard for classifying autophagic cell death. Unlike apoptosis, caspases are not activated and therefore DNA degradation is minimal, the nucleus remains intact, and cellular fragmentation is not observed (111). Membrane blebbing however, does occur and seems to be a common feature of apoptosis and autophagy (111, 153). For the most part, autophagic cells are cleared by self-ingestion mediated by lysosomal hydrolases within the autolysosome and are occasionally cleared by phagocytes (111). As expected, the controlled removal of autophagic cells does not activate the inflammatory response (111).

1.2.3 Necrosis

Necrotic cell death has a number of morphological and biochemical features that make it unique from apoptosis and autophagy (Table 1-3). Classical necrosis is defined as a passive form of cell death that is induced by pathogenic states including trauma, infection and ischemia (149, 193). A more refined definition states that necrosis is a result of bioenergetic catastrophe in which a cell is no longer able to produce sufficient ATP for survival (80, 168). Cells entering into necrosis swell, and eventually lyse, releasing their cellular contents and in the process, provoking an inflammatory response (259). In the past, necrosis was thought to be an uncontrolled cell death process however, recent evidence suggests cells undergo a programmed version of necrosis. High levels of reactive oxygen species (129), serine protease activity (231), and DNA alkylation (374) are all triggers for programmed necrosis. Although the biochemical mechanisms have yet to be defined for programmed necrosis, recent evidence indicates a mitochondrial pathway of necrosis that is dependent on the opening of the permeability transition pore (PTP), a specific non-selective high-conductance protein-containing megachannel located at the contact site between the inner and outer mitochondrial membrane (15, 35, 221). Interestingly, overexpression of anti-apoptotic proteins including Bcl-2 and Bcl-XL have been shown to protect cells from necrosis suggesting that necrosis may be inhibited under certain conditions (288). Programmed necrosis can morphologically resemble classical necrosis or alternatively overlap with the other types of cell death, in particular autophagy (79, 143). For example, autophagic cells no longer capable of generating ATP have been shown to switch to an autophagic/necrotic version of programmed cell death characterized by the appearance of autophagosomes and the breakdown of the plasma membrane and activation of the inflammatory response (79).

1.3 Caspases

Caspases constitute a family of cysteine proteases that share a strong stringency for cleaving their substrates after aspartate residues (7). A critical role for caspases in apoptosis was initially discovered during studies with the nematode *Caenorhabditis elegans*. Using a genetic screen approach, Horvitz and colleagues demonstrated that the cysteine protease CED-3 (cell-death abnormality-3) which shares homology to the first identified caspase, IL-1β converting enzyme (ICE), was necessary for apoptosis that occurred during development (367). Caspases are now regarded as central coordinators of apoptosis and inflammation. Currently, fourteen mammalian caspases have been identified with orthologs present in organisms ranging from mammals to metazoans (4, 163, 192, 321, 360). Of the fourteen mammalian caspases, eleven are encoded by the human genome (321).

The caspase family can be subdivided into three families based on their sequence similarities, function, and substrate specificity (Figure 1-3). To date, seven caspases serve as central mediators of apoptosis and these can be further divided into 'initiator' and 'executioner' caspases based on their order of activation during apoptosis (Figure 1-3) (50, 54, 78, 100, 181). Initiator caspases (caspase-2, -8, -9, and -10) are characterized by an extended N-terminal region (>90 amino acids) that includes at least one homotypic caspase-recruitment domain (CARD) or death effector domain (DED). Executioner caspases (caspase-3, -6, and -7), which account for the morphological features associated with apoptosis, contain a prodomain no more than 30 amino acids in length and are activated by initiator caspases (96).

1.3.1 Mechanism of Caspase Activation

In healthy cells, all caspases exist as inactive zymogens containing an N-terminal prodomain, followed by large p20 and small p10 catalytic subunit domains separated by a linker region (Figure 1-4A). A fully functional caspase is a homodimer that contains two active sites. Each homodimer consists of one large and one small subunit. Historically, activation of initiator and executioner caspases was thought to be regulated through a series of cleavage events. Although this appears to be true for executioner caspases, recent evidence indicates that initiator caspase activation occurs by a distinct mechanism (16, 30, 74, 306).

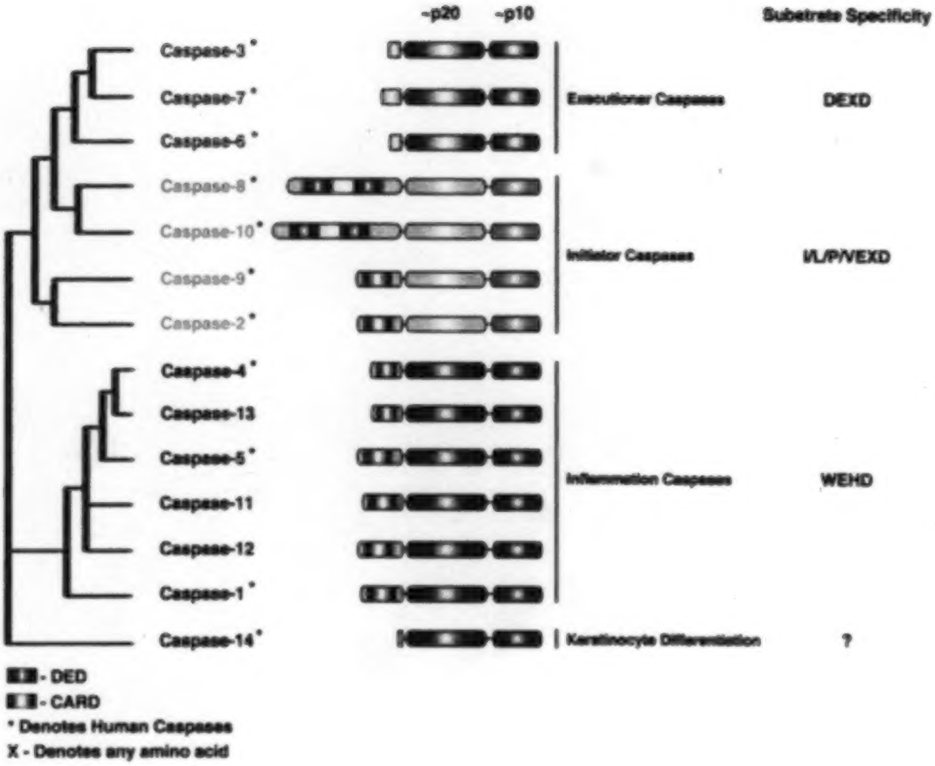

~p20
~p10
Substrate Specificity
Caspase-3 *
Caspase-7 *
Caspase-6 *
Caspase-8 *
Caspase-10 *
Caspase-9 *
Caspase-2 *
Caspase-4 *
Caspase-13
Caspase-5 *
Caspase-11
Caspase-12
Caspase-1 *
Caspase-14 *
Executioner Caspases
DEXD
Initiator Caspases
I/L/P/VEXD
Inflammation Caspases
WEHD
Keratinocyte Differentiation
?
- DED
- CARD
* Denotes Human Caspases
X - Denotes any amino acid

Figure 1-3 Phylogenic analysis, substrate specificity and function of mammalian caspases.

Caspases can be subdivided into three subfamilies based on their sequence identity. Caspase substrate specificity and function correlates with the phylogenic relationship between caspases. Adapted from Shi, Y 2002 (287).

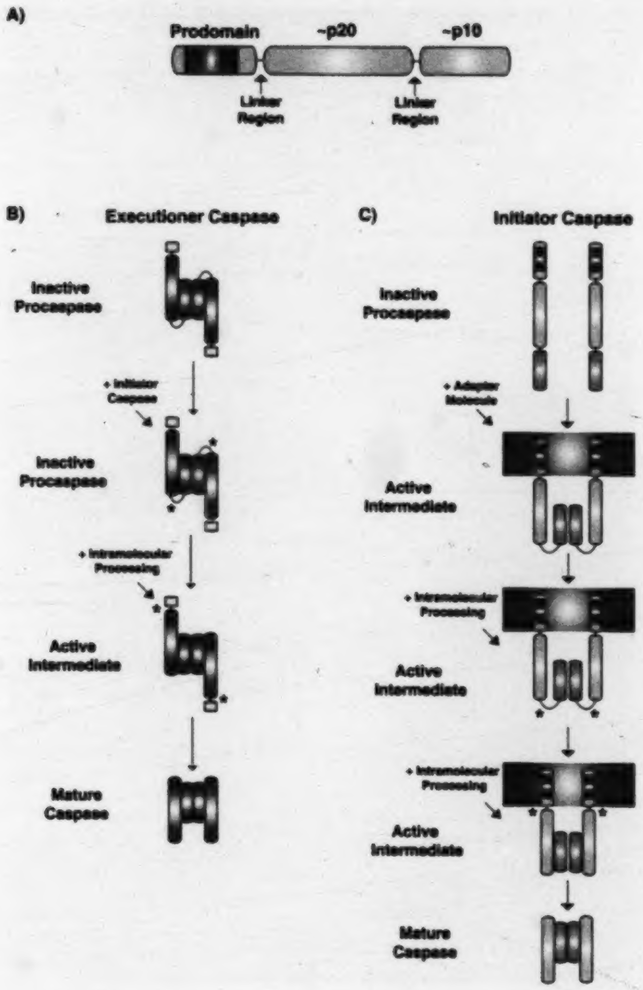

A)
Prodomain
~p20
~p10
Linker Region
Linker Region
B)
Executioner Caspase
Inactive Procaspase
+ Initiator Caspase
Inactive Procaspase
+ Intramolecular Processing
Active Intermediate
Mature Caspase
C)
Initiator Caspase
Inactive Procaspase
+ Adapter Molecule
Active Intermediate
+ Intramolecular Processing
Active Intermediate
+ Intramolecular Processing
Active Intermediate
Mature Caspase

Figure 1-4 Mechanisms of caspase activation.

(A) Caspases are synthesized as inactive zymogens. (B and C) Caspases require proteolytic processing to become fully active. (B) Executioner caspases are present as homodimers within cells. Activation of executioner caspases requires a trans-cleavage event by an initiator caspase leading to partial activation. (*) denotes proteolytic cleavage after aspartate residues in linker regions. Full activation is achieved by intramolecular cleavage that removes the prodomain. (C) Initiator caspases are monomeric in healthy cells. Following an apoptotic stimulus, adapter proteins mediate caspase dimerization and activation. Dimerization is followed by cleavage between the large and small subunit to generate a stable active caspase. Further processing removes the prodomain and releases the active caspase from adapter proteins.

Under non-apoptotic conditions, executioner procaspases exist as inactive homodimers with no inherent enzyme activity (30). Executioner caspase activation requires an intrachain cleavage within the linker region separating the large and small subunit that is carried out by an initiator caspase (Figure 1-4B) (92, 200). This initial cleavage generates a partially active catalytic intermediate that proceeds to remove the prodomain from the large subunit to generate a fully active caspase (92, 200).

A unified model has recently been described for the activation of initiator caspases which proposes that dimerization of initiator caspases determines their activation (Figure 1-4C) (16, 30, 74, 306). Unlike executioner caspases, initiator caspases exist as inactive monomers in healthy cells (30). In response to a proapoptotic signal, initiator procaspases dimerize with the help of an adapter molecule to assume a conformation that is partially active. Although not required for caspase activity, *in trans* proteolytic processing occurs between the large and small subunit to generate a fully active homodimer due to complex stabilization (269). Further processing releases the fully mature homodimeric caspase from the activation complex.

Caspase substrate specificity is mainly determined by four N-terminal amino acid residues immediately upstream of the scissile cleavage bond referred to as P4-P3-P2-P1 (321). The P1 position always contains aspartate however, the other three substrate position vary in amino acid content and are the main contributers to caspase substrate specificity. In general, caspases can be grouped into three categories based on their optimal substrate recognition sequences (Figure 1-3) (258, 322). Group I caspases, which include all the inflammatory caspases, prefer bulky hydrophobic side chains at the P4 substrate position and prefer the WEHD tetrapeptide (258, 322). Group II caspases, which include executioner caspases, preferentially incorporate DEXD (where X is any amino acid) tetrapeptides. Group III caspases, which includes all initiator caspases preferentially bind tetrapeptides with aliphatic amino acids at the P4 substrate position and prefer I/L/P/VEXD (where X is any amino acid) tetrapeptides (258, 322).

1.3.2 Negative Regulation of Caspases

The enzymatic activity of caspases is under strict regulation by a conserved family of inhibitor of apoptosis (IAP) proteins (71). The first IAP discovered was encoded by baculovirus and was found to inhibit apoptosis in virus infected cells (58). Proteins with homology to baculovirus IAP have been identified in other viruses, yeast, worms, flies and mammals (334). Currently, eight mammalian IAPs have been identified all of which are able to inhibit caspase activity either directly or indirectly and to different degrees and specificities (71, 270).

All IAPs contain one to three zinc binding baculovirus IAP repeat (BIR) motifs that are approximately 70 amino in length and essential for the inhibition of apoptosis (334). Structural and biochemical studies with X-linked IAP (XIAP), the prototypical member of the IAP family, have revealed the mechanism of IAP mediated inhibition of caspases (44, 141, 264, 285, 294). XIAP contains three BIR domains and inhibits initiator (caspase-9) and executioner (caspase-3 and -7) caspase activity by distinct mechanisms. The BIR3 region of XIAP inhibits caspase-9 dimerization by interacting with two distinct interfaces within caspase-9 that sequesters the caspase in an inactive monomeric state (294, 305, 309). The mechanism by which XIAP binds to caspase-3 and -7 reflects that executioner caspases are natural homodimers in healthy cells. Following the initial cleavage of a procaspase, the BIR2 region and a short linker region that precedes the BIR2 region form protein interfaces with active caspase-3 and -7 which function to block the substrate binding pocket to inhibit executioner caspase function (44, 72, 141, 264, 285, 309).

IAPs are not excluded from molecular regulation. Both mammalian and insect IAPs are regulated by a growing family of proteins known as IAP binding motif (IBM) proteins (26, 341). In mammals, IBM proteins include second mitochondrial activator of caspases/direct IAP-binding protein with low PI (SMAC/Diablo) and high temperature required protein A (HtrA2) also referred to as Omi, that are sequestered in mitochondria until an apoptotic stimulus is generated (26, 72, 309, 341). In contrast, insect IBM proteins including Grim/Hid/Reaper are transcriptionally regulated (26, 341). IBM proteins contain an N-terminal tetrapeptide motif that binds to a conserved hydrophobic pocket located on the surface of IAP BIR domains (26, 341). The interaction between IBM and IAP proteins inhibits IAP-mediated caspase regulation thereby allowing the apoptosis to proceed.

1.4 Apoptotic Pathways

Apoptosis is initiated by many different types of stimuli. Each stimulus induces cell death by one of three unique pathways that feed into a conserved caspase dependent signalling pathway. The majority of apoptotic cascades proceed through the intrinsic pathway whereas the extrinsic and granule mediated apoptotic pathways are utilized by immune system, in particular cytotoxic T cells (CTL) and natural killer cells (NK), to remove damaged cells, virus infected cells and maintain immune system homeostasis.

1.4.1 The Intrinsic Apoptosis Pathway

The intrinsic apoptotic pathway is initiated from within the cell and absolutely requires the involvement of the mitochondria to induce cellular death (Figure 1-5). Apoptosis induced by DNA damage, growth factor withdrawal, organelle stress and pathogen invasion results in the mitochondria undergoing a number of structural and physiological changes (70, 161, 202, 352). These changes include the selective permeabilization of the outer mitochondrial membrane and the release of pro-apoptotic proteins such as cytochrome c, SMAC/Diablo, HtrA2/Omi, endonuclease G (EndoG), and apoptosis inducing factor (AIF) into the cytosol (See section 1.5.2) (75, 174, 183, 312, 315). The release of cytochrome c is a pivotal step in the apoptotic pathway and serves to induce a conformational change in apoptosis protease-activating factor-1 (Apaf-1) that leads to activation of caspase-9 (Figure 1-5) (175). Under non-apoptotic conditions, Apaf-1 is a globular molecule that consists of WD40 repeats and a CARD domain (1). Upon release into the cytoplasm, cytochrome c interacts with Apaf-1 via its WD40 repeats (1). Cytochrome c binding to Apaf-1 induces the conformational change and polymerization of Apaf-1 in the presence of dATP/ATP to form the apoptosome (175). The open oligomerized conformation of Apaf-1 forms the core of the apoptosome and recruits caspase-9 via its N-terminal CARD domain inducing the formation of caspase-9 dimers (378). The subsequent activation of caspase-9 triggers downstream activation of executioner caspases to generate apoptotic cell death.

1.4.2 The Extrinsic Apoptotic Pathway

The extrinsic pathway is initiated through the binding of ligands to death receptors. Death receptors are type I transmembrane proteins that include Fas(CD95),

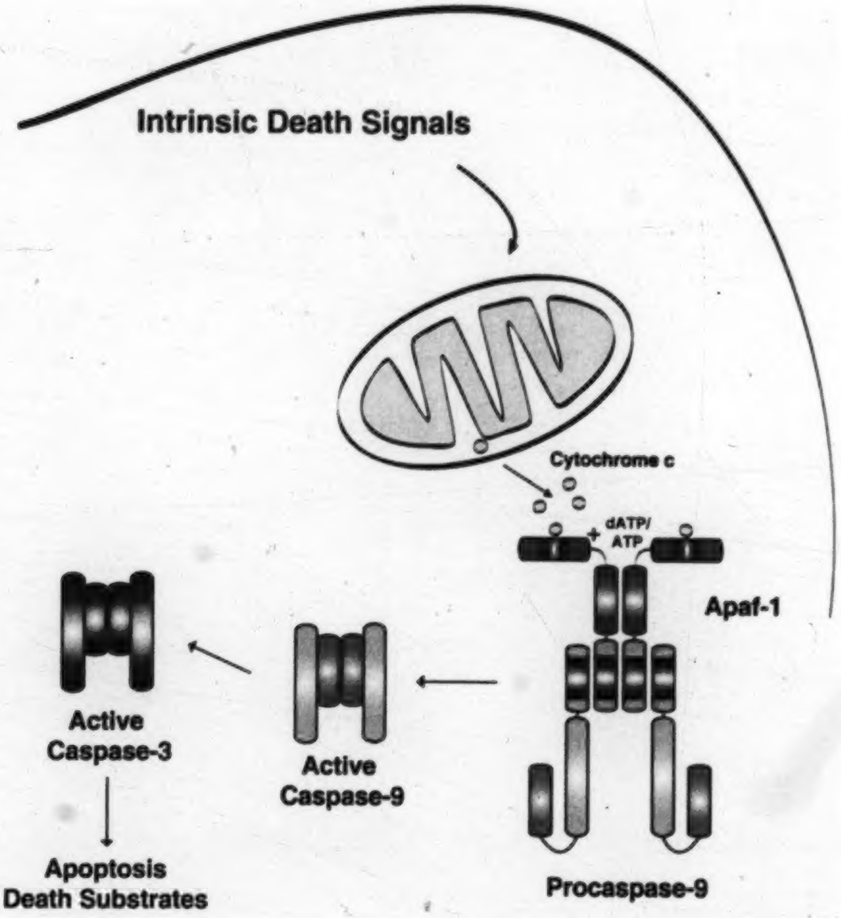

Intrinsic Death Signals
Cytochrome c
dATP/
ATP
Apaf-1
Active
Caspase-3
Active
Caspase-9
Procaspase-9
Apoptosis
Death Substrates

Figure 1-5 Schematic representation of the intrinsic apoptotic cascade. Intracellular death signals proceed through the mitochondria which is a critical regulatory checkpoint. In response to an apoptotic stimulus, mitochondria release cytochrome c that binds to Apaf-1 and induces its oligomerization in the presence of dATP/ATP. Oligomerized Apaf-1 serves as an activation station for caspase-9. Once activated, caspase-9 proteolytically cleaves caspase-3 that proceeds to cleave cellular substrates resulting in cellular apoptosis.

tumour necrosis factor receptor 1 (TNFR1), and death receptors -4 and -5 (DR4/5) (3, 245). Members of this family share five cysteine-rich repeats in their extracellular domain as well as a cytoplasmic homotypic death domain (DD) that is absolutely required for transduction of the death signal (3, 245). Each death receptor utilizes a unique ligand to trigger apoptosis. In the case of Fas receptor, apoptosis is activated upon engagement with Fas ligand (FasL) (308). Activation of apoptosis with TNFR1 requires its ligand TNFα (318). Both DR4 and DR5 share a common ligand known as Apo2L/TRAIL(TNF-Related Apoptosis Inducing Ligand) (12).

One of the best studied death receptor pathways is the Fas receptor (Figure 1-6). Fas receptor is activated by FasL which induces a conformational change in Fas receptor that results in the formation of receptor microaggregates (6). The adapter protein Fas-associated death domain (FADD) contains a DD that binds to the DD of Fas to initiate the formation of the death inducing signalling complex (DISC) (51, 155). FADD also contains a DED and recruits the DED containing procaspase-8 into the DISC resulting in the dimerization and activation of procaspase-8 (32, 219). The levels of activated caspase-8 predispose the next series of apoptotic events. In type I cells, large amounts of caspase-8 are activated followed by rapid cleavage of caspase-3 and other executioner caspases (274). In type II cells however, a low amount of caspase-8 is activated necessitating the need for an amplification of the apoptotic signal (274). Under this circumstance caspase-8 stimulates the intrinsic cascade by cleaving the BH3-only Bcl-2 family protein Bid, inducing the C-terminus of Bid (tBid) to translocate to mitochondria and activate the intrinsic apoptosis pathway (173, 189). It is important to note that mitochondria are involved in both type I and type II apoptotic cascades. Whereas

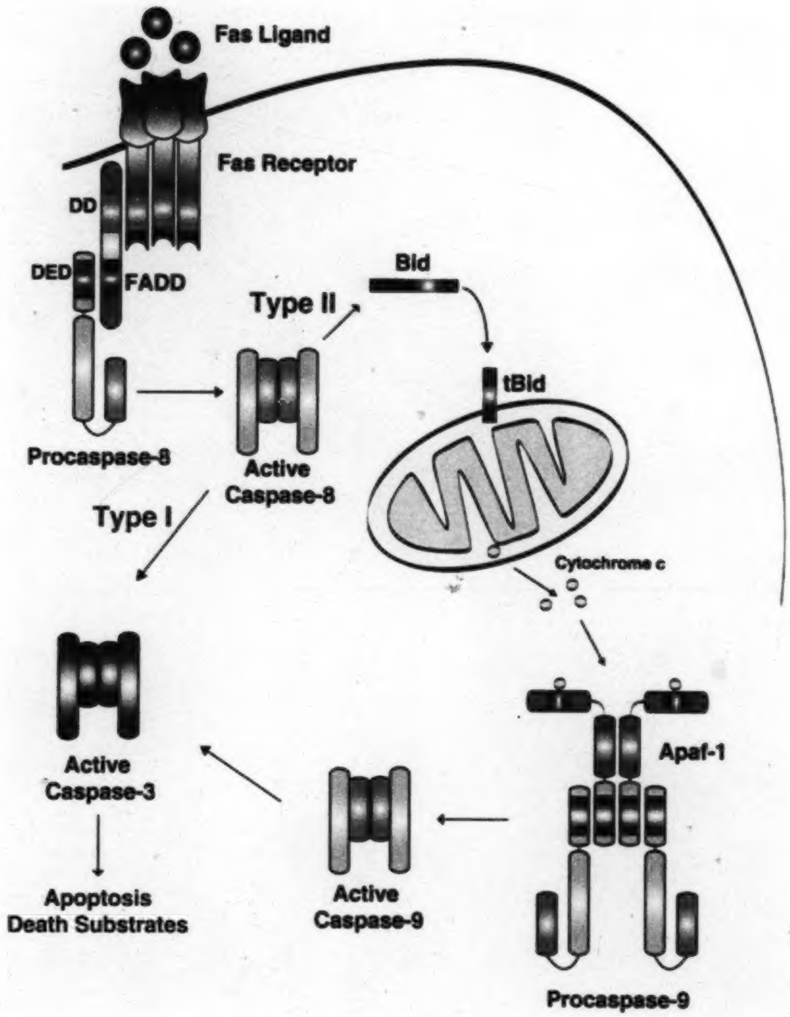

Fas Ligand
Fas Receptor
DD
DED
FADD
Type II
Bid
tBid
Procaspase-8
Active Caspase-8
Type I
Cytochrome c
Apaf-1
Active Caspase-3
Apoptosis Death Substrates
Active Caspase-9
Procaspase-9

Figure 1-6 Schematic representation of the Fas receptor-mediated extrinsic apoptotic cascade.
The extrinsic apoptotic cascade is triggered by death receptors. One of the best understood pathways is the FasL-Fas receptor cascade. Upon interaction with its ligand, Fas receptor induces formation of the DISC and activation of caspase-8. In type I cells, active caspase-8 cleave caspase-3. In type II cells, active caspase-8 triggers the intrinsic cascade by cleaving and activation Bid.

mitochondria are absolutely necessary for apoptosis in type II cells, mitochondria serve to amplify the death signal in type I cells.

1.4.3 The Granzyme B Apoptosis Pathway

NK and CTL utilize lytic granules for the delivery of death signals to virus infected and damaged cells. Granules contain proteolytic enzymes that induce alternative forms of cell death when introduced independently into a cell (326). Of these, granzyme B is the only granule enzyme that cleaves substrates after aspartate residues and activates apoptotic pathways (66, 67, 123, 200, 256, 322).

Once released into the cytoplasm of target cells, granzyme B preferentially activates the mitochondrial apoptotic pathway by one of two cleavage events (Figure 1-7). Granzyme B induces apoptosis by directly cleaving Bid prompting activation of the intrinsic pathway (19, 127, 128). Recently, the pro-survival Bcl-2 family member Mcl-1 was identified as a new granzyme B substrate (120). In healthy cells, Mcl-1 sequesters the pro-apoptotic protein Bim in an inactive complex (120). Cleavage of Mcl-1 by granzyme B liberates Bim from Mcl-1 regulation resulting in the release of pro-apoptotic proteins from the mitochondria (120, 121). Previous work implicated granzyme B in initiating apoptosis by cleaving and activating caspase-3 (66, 67, 256). Granzyme B cleaves caspase-3 to its p20 and p10 fragments however full caspase-3 activation requires the additional removal of XIAP (109, 214). Granzyme B induced activation of the intrinsic apoptotic cascade triggers the release of SMAC/Diablo from the mitochondria that liberates caspase-3 from the inhibitory effects of XIAP (109, 214). Once free from XIAP regulation, caspase-3 catalytically processes itself to a fully active form (200).

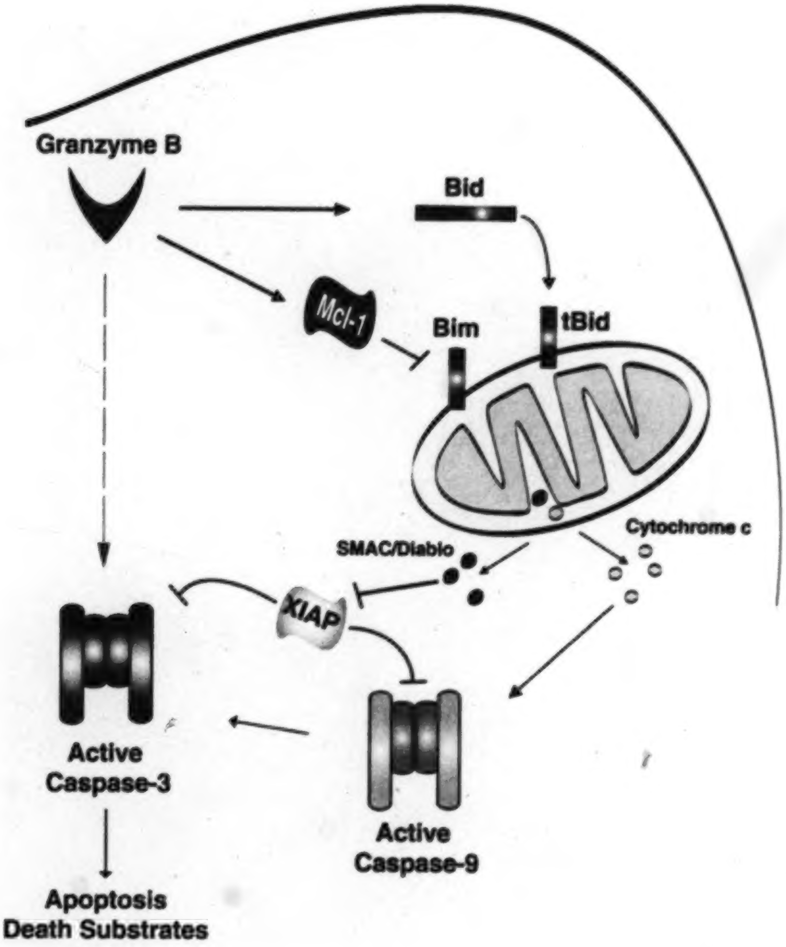

Granzyme B
Bid
Mcl-1
Bim
tBid
SMAC/Diablo
Cytochrome c
XIAP
Active Caspase-3
Active Caspase-9
Apoptosis Death Substrates

Figure 1-7 Schematic representation of the granzyme B-mediated apoptotic cascade. When introduced into a cell, granzyme B partially activates caspase-3 and activates the intrinsic apoptotic cascade by one of two cleavage events. Granzyme B cleaves Bid to promote the translocation of tBid to mitochondria or cleaves and inactivates Mcl-1 thereby activating Bim. Bim and tBid induce the release of cytochrome c to activate caspase-9. The partially active form of caspase-3 generated by granzyme B cleavage is liberated from XIAP regulation by the release of SMAC/Diablo.

1.5 Mitochondria and Apoptosis

A number of observations in the early 1990s implicated mitochondria in apoptosis. Reed and Newmeyer observed that mitochondrial enriched fractions were required for nuclear apoptosis to occur in *Xenopus* egg extracts (247, 342, 369). Multiple groups observed the loss of the inner mitochondrial membrane potential, the electric potential difference across the inner mitochondrial membrane, as an early event that correlated with DNA fragmentation (206, 222, 225). A novel oncogene, Bcl-2, localized to membrane fractions including mitochondrial membranes and inhibited apoptosis (132, 225). However, it wasn't until Wang and colleagues discovered the role of cytosolic holo-cytochrome c in caspase activation that the skeptics were convinced (175, 183). Today, the function of mitochondria and its role in cell death continues to be the focus of intense research and debate.

1.5.1 Mitochondrial Structure and Function

Mitochondria are unique organelles in that they carry multiple copies of a circular DNA genome as well as the necessary transcriptional and translational machinery to generate proteins (186). While the mitochondrial genome varies in size among organisms all mitochondrial genomes encode small and large ribosomal subunits, twenty-two aminoacyl tRNAs, and thirteen polypeptides that encode subunits of the oxidative phosphorylation pathway (186). This constitutes only a small percentage of mitochondrial proteins as the majority of mitochondrial proteins are encoded by nuclear genes.

In recent years, the perception of mitochondrial structure has been redefined due in large part to high-voltage electron microscopy (HVEM) technology (99, 195). Each mitochondrion is composed of two highly specialized lipid membranes with differential protein and lipid content. The outer membrane is a static molecular sieve that allows passive transfer of molecules <5kDa, predominantly through the voltage dependant anion channel (VDAC). The inner membrane consists of two compartments, the inner boundary membrane that is closely adjacent to the outer membrane and the cristae membrane that forms invaginations into the matrix (Figure 1-8A). These two compartments are separated by narrow tubular segments called cristae junctions that are predicted to be approximately 28nm in diameter(98, 99). The exact role of the cristae junctions is unclear, however, it has been postulated that they act as molecular barriers to regulate the diffusion of proteins and/or metabolites within the intermembrane space (195, 196). The inner membrane is constantly responding to environmental changes by either forming or collapsing cristae junctions (197). In contrast to the outer membrane, the inner membrane is strictly impermeable to the passive transfer of most molecules and relies on specific substrate/ion transporters that allow for the exchange of ions, respiratory substrates and products (206). The strict permeability of the inner membrane is central to the generation of ATP by a process referred to as oxidative phosphorylation (206). Oxidative phosphorylation involves the movement of electrons through the inner membrane that is coupled to the generation of ATP (Figure 1-8B). Embedded within the inner mitochondrial membrane are electron carriers that assemble to form five multiprotein complexes that receive and pass electrons ending with the reduction of O_2. Complex I and complex II receive electrons by oxidizing electon carriers NADH (reduced nicotinamide adenine dinucleotide) and $FADH_2$ (reduced flavin adenine dinucleotide) respectively. In turn, the electrons are passed through complex III and

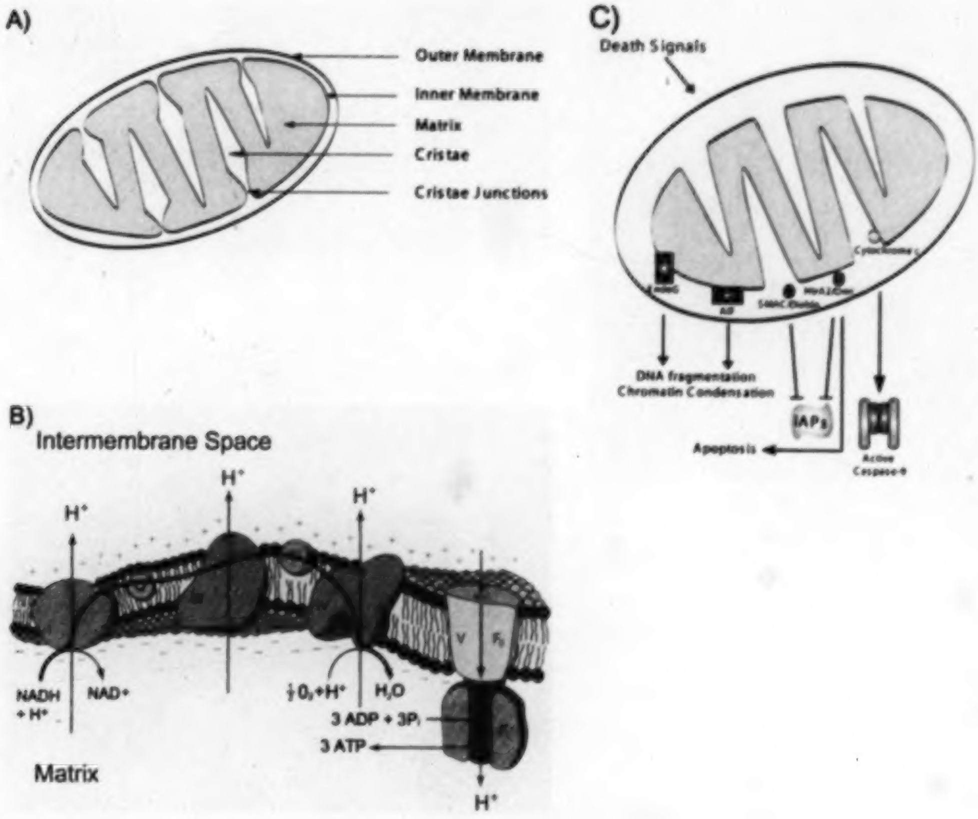

A)
Outer Membrane
Inner Membrane
Matrix
Cristae
Cristae Junctions
C)
Death Signals
EndoG
AIF
SMAC/Diablo
HtrA2/Omi
Cytochrome c
DNA fragmentation
Chromatin Condensation
IAPs
Apoptosis
Active Caspase-9
B)
Intermembrane Space
H+
H+
H+
V
F0
NADH + H+
NAD+
½ O2+H+
H2O
3 ADP + 3Pi
3 ATP
Matrix
H+

Figure 1-8 Mitochondrial structure and pro-apoptotic proteins released during apoptosis.

(A) Cross section of mitochondria. (B) Schematic representation of the mitochondrial electron transport chain. Electrons are transferred from NADH to oxygen (red line) by electron carriers that is coupled to ATP production. As electrons move through the electron transport chain, H^+ are pumped from the matrix into the intermembrane space through complex I, III and IV, generating an electrochemical gradient that provides energy to drive ATP synthesis. ATP is produced when H^+ enters the matrix through complex V. Electrons transferred from $FADH_2$ enter the electron transport chain through complex II (not shown) resulting in ATP generation without the involvement of complex I. Adapted from Mathews, CK and van Holde KE 1996 (206). (C) Pro-apoptotic proteins that normally reside in healthy mitochondria are released following an apoptotic stimulus. Each pro-apoptotic protein is differentially retained in the intermembrane space. EndoG and AIF are either inserted or strongly associated with the inner membrane. SMAC/Diablo and HtrA2/Omi are soluble and cytochrome c is bound to the inner membrane by electrostatic interactions. Following release from the mitochondria, each pro-apoptotic protein contributes to the induction of apoptosis by unique mechanisms.

complex IV via electron carriers. During the movement of electrons, H^+ is pumped from the matrix to the intermembrane space through protein complexes I, III, and IV that results in a proton concentration gradient and electric potential difference referred to as the electrochemical gradient. The electrochemical gradient drives ATP synthesis when protons enter the matrix via an ATPase (complex V) to generate ATP from ADP and inorganic phosphate. Collectively, the impermeability of the inner mitochondrial membrane and the electrochemical gradient are two physiological features of the mitochondria that are absolutely necessary for ATP production.

1.5.2 Mitochondrial Permeabilization during Apoptosis

Numerous cell death signals converge at the mitochondria to initiate cell death. The permeabilization of the outer membrane is a pivotal step during apoptosis and results in the release of many proteins from the intermembrane space (238, 336). Some of these proteins including cytochrome c, SMAC/Diablo, HtrA2/OMI, EndoG, and AIF have pro-apoptotic functions (Figure 1-9C). Cytochrome c was the first mitochondrial pro-apoptotic protein to be identified (183). Once released into the cytoplasm, cytochrome c activates caspase-9 leading to the subsequent activation of executioner caspases to induce apoptosis (175). Cytochrome c also triggers calcium release from the ER through its direct interaction with inositol (1,4,5) triphosphate receptor (31). Cytoplasmic calcium mediates additional cytochrome c release from mitochondria to enhance the initial apoptotic signal (31). Once released into the cytoplasm, SMAC/Diablo antagonizes the caspase inhibitory action of IAPs to liberate catalytically active caspases (75, 343). Similar to SMAC/Diablo, the serine protease HtrA2/Omi outcompetes caspases for IAP binding (126, 315, 344). HtrA2/OMI also contributes to caspase-independent cell death independent of its IAP binding function but dependent on its serine protease activity (126, 315, 344). When released into the cytoplasm, EndoG migrates to the nucleus and induces caspase-independent intranucleosomal DNA fragmentation, likely in concert with exonucleases and DNase I (174, 358). Similar to EndoG, AIF also migrates to the nucleus where it directly promotes chromatin condensation and higher order large-scale DNA fragmentation in the absence of caspase activity (312, 313). In parallel with the functional disparity of the above pro-apoptotic proteins, the kinetics for the release of these intermembrane proteins are likely different as well (335). Whereas SMAC/Diablo and HtrA2/Omi are soluble, cytochrome c is bound to the phospholipid inner membrane by strong electrostatic charges while EndoG and AIF are tethered to or found within the inner membrane (335).

1.5.3 Bcl-2 Family Members Regulate Apoptosis at the Mitochondria

Bcl-2 family members tightly regulate apoptosis at the mitochondria. The founding member, Bcl-2, was initially identified as a proto-oncogene that, unlike other oncogenes at the time, did not promote cell proliferation but instead inhibited cell death (210, 340). Members of the Bcl-2 family have been identified in all metazoan organisms studied to date (166, 359). The proteins are characterized as either pro- or anti-apoptotic and bear one or more distinct functional domains called Bcl-2 homology (BH) domains (BH1 to BH4) (Figure 1-9) (56, 117). Some family members also contain a carboxyl-terminal transmembrane region that promotes membrane localization and insertion (277). Most members of the anti-apoptotic subclass possess all four BH domains and include Bcl-2, Bcl-XL, Bcl-w, A1/Bfl-1, and Boo/Diva (56). Mcl-1, on the other hand, contains

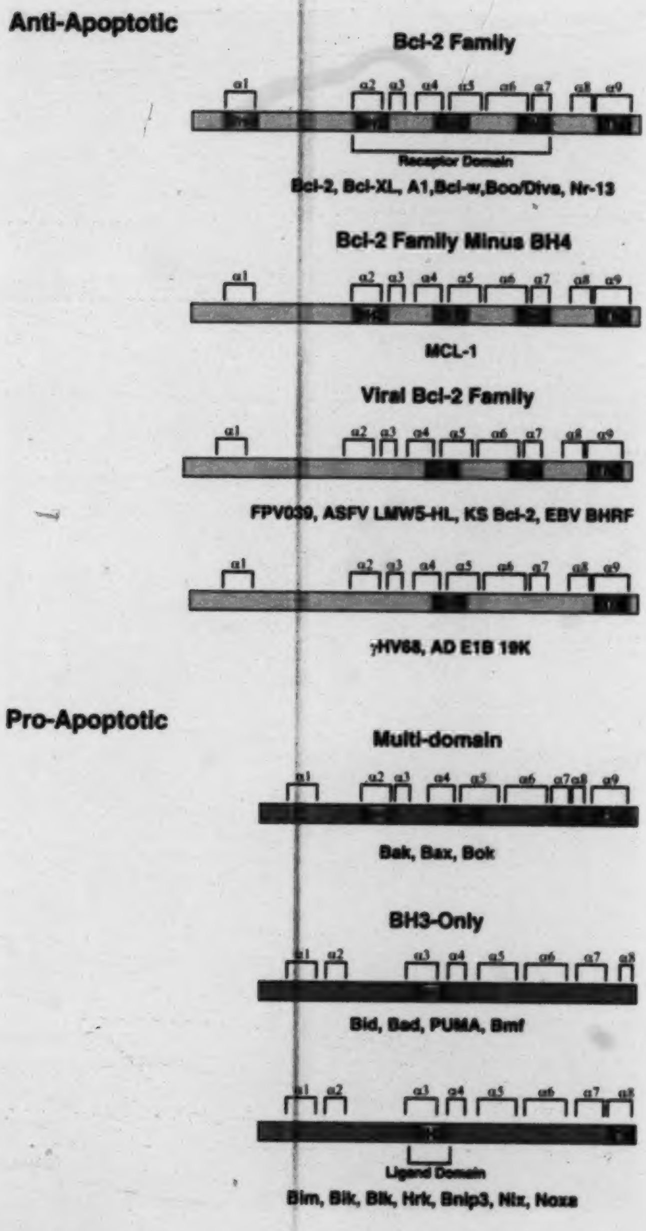

Anti-Apoptotic
Bcl-2 Family
Receptor Domain
Bcl-2, Bcl-XL, A1,Bcl-w,Boo/Diva, Nr-13
Bcl-2 Family Minus BH4
MCL-1
Viral Bcl-2 Family
FPV039, ASFV LMW5-HL, KS Bcl-2, EBV BHRF
γHV68, AD E1B 19K
Pro-Apoptotic
Multi-domain
Bak, Bax, Bok
BH3-Only
Bid, Bad, PUMA, Bmf
Ligand Domain
Bim, Bik, Blk, Hrk, Bnip3, Nix, Noxa

Figure 1-9 The Bcl-2 family.

Members of the Bcl-2 family contain at least one BH domain and are subdivided into anti- and pro-apoptotic proteins. Many family members contain a C-terminal transmembrane (TM) domain that promotes membrane localization. AD, adenovirus; ASFV, African swine fever virus; EBV, Epstein-Barr virus; FPV, fowlpox virus; γHV, gammaherpesvirus; KS, Kaposi sarcoma herpesvirus.

a BH1, BH2, and BH3 domain but lacks a region with strong homology to the BH4 domain (159). Most viral encoded anti-apoptotic Bcl-2 (vBcl-2) proteins contain BH1 and BH2 domains only (61, 122). Members of the pro-apoptotic family including Bak, Bax, and Bok possess BH1, BH2, and BH3 domains and are referred to as the multi-domain pro-apoptotic family members (55, 117). An additional set of pro-apoptotic family members that contain only a BH3 domain include Bid, Bad, Bik, Blk, Hrk, Bim, Bnip3, Nix, Noxa, PUMA, and Bmf and are referred to as BH3-only proteins (55, 117).

Exactly how Bcl-2 proteins regulate apoptosis is unclear. Genetic and biochemical studies suggest that Bak and Bax are essential for outer mitochondrial membrane permeabilization and that BH3-only proteins are necessary for their pro-apoptotic function while anti-apoptotic members antagonize both multi-domain and BH3-only pro-apoptotic family members (277, 375).

1.5.3.1 Structural properties of Bcl-2 proteins

The structure Bcl-2, Bcl-XL, and viral Bcl-2 like proteins from Kaposi's sarcoma associated virus (KSHV) and Epstein-Barr virus as well as pro-apoptotic Bax and Bid have been solved (139, 140, 218, 248). Structural analysis indicates a similar fold pattern irrespective of whether the protein has pro- or anti-apoptotic function. Overall, the structure consists of two central hydrophobic hairpin α-helices surrounded by six or seven amphipathic α-helices that can vary in length (Figure 1-10). Between α-helices one and two is an unstructured loop that also differs in length. A distinct hydrophobic groove is generated by the close proximity of the BH1, BH2, and BH3 domains and serves as a binding site for the amphipathic BH3 regions from pro-apoptotic Bcl-2 family

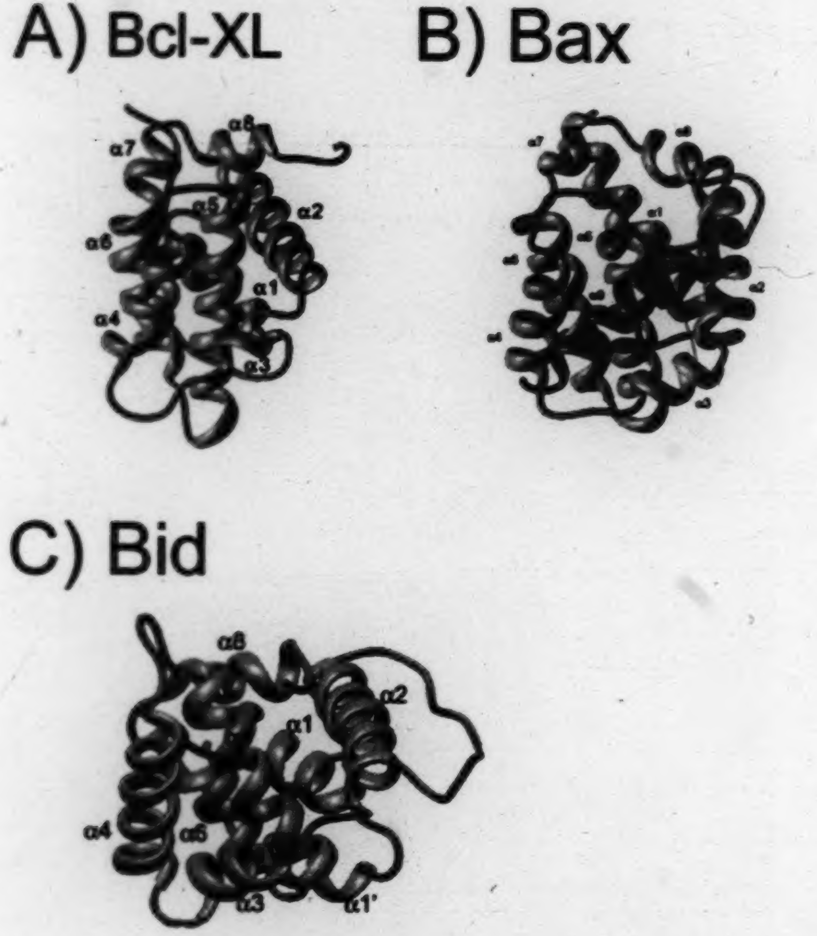

A) Bcl-XL
B) Bax
C) Bid
α8
α7
α5
α2
α6
α1
α4
α3
α8
α2
α1
α4
α6
α3
α1'

Figure 1-10 Ribbon representation of Bcl-2 family members.
Ribbon representation of anti-apoptotic Bcl-XL (A), pro-apoptotic Bax (B), and BH3-only protein Bid. Helices in Bax and Bid are numbered with respect to Bcl-XL. Of note, helix 9 in Bax (shown in red) corresponds to the transmembrane helix of Bcl-XL and occupies the hydrophobic pocket of Bax. Bid contains an extra helix denoted α1'. Adapted from Petros, AM. *Et al.* 2004 (249).

proteins (182, 272). The structure of pro-apoptotic Bax also demonstrates the presence of a hydrophobic groove generated by the close proximity of BH1, BH2, and BH3 domains however, the groove is blocked by the C-terminal transmembrane region which becomes exposed following an apoptotic stimulus (314). Although Bid contains only a BH3 only domain, its structure is also similar to other Bcl-2 family proteins with one significant difference: the hydrophobic region on the surface is flat and lacks even a subtle hydrophobic groove (209). Structurally, Bcl-2 proteins resemble the membrane spanning region of bacterial pore-forming toxins including colicin and diphtheria (Figure 1-11) (25, 218, 237). This homology includes the two central α-helices that are surrounded by a number of amphipathic α-helices. This observation has led to the theory that Bcl-2 proteins are capable of forming membranous pores that allow the passage of ions or proteins (275, 276, 279). Indeed, evidence indicates the Bcl-2, Bcl-XL, Bid and Bax all exhibit ion channel activities when incorporated into lipid bilayers or liposomes and that this activity is dependent on the two central helices (α-helices 5 and 6) (275, 276, 279).

1.5.3.2 BH3–only proteins: sentinels of apoptotic signals

Specific cell death signals are transduced to the mitochondrial checkpoint primarily by BH3-only proteins (138). Individual BH3-only proteins are responsible for inducing apoptosis in response to specific signals although some overlap exists (138). The activation of the BH3-only proteins is tightly regulated either by de novo transcription or post-translational modifications such as phosphorylation and proteolytic cleavage (138, 254). For example, the BH3-only protein Bid is a soluble globular protein that resides in the cytoplasm in healthy cells and is an essential component of death

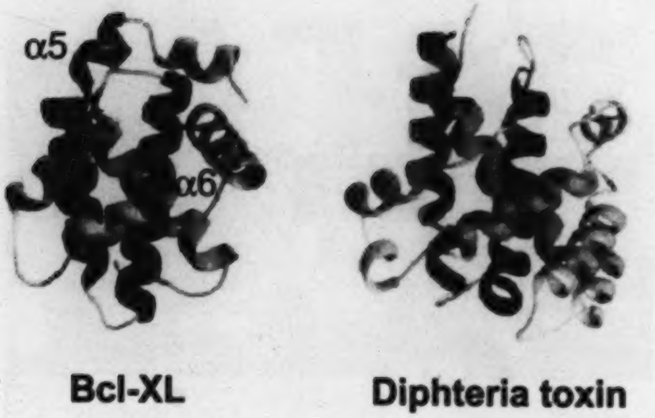


Figure 1-11 Structural similarities of Bcl-XL and diphtheria toxin.
Comparison of the solution structure reveals a distinct similarity in structure between Bcl-XL and the pore forming domain of the diphtheria toxin. The homology includes two central α-helices (α5/6) in Bcl-XL that is highly similar to the membrane spanning region in diphtheria toxin. Surrounding the two central helices (shown in red) is additional amphipathic α-helices (shown in blue and grey). Adapted from Muchmore, SW. *et al.* 1996 (218).

receptor mediated apoptosis in type II cells and granzyme B induced apoptosis (see section 1.4.1 and 1.4.2). Proteolytic cleavage of Bid by either caspase-8 or granzyme B produces tBid which rapidly translocates to mitochondria and triggers cytochrome c release (19, 189).

The BH3-only proteins are proposed to induce apoptosis by either binding and antagonising the activity of anti-apoptotic Bcl-2 family proteins or binding and activating pro-apoptotic multi-domain Bcl-2 family proteins (49, 169, 375). The hydrophobic face of the amphipathic α-helix BH3 domain is necessary to achieve the pro-apoptotic function of BH3-only proteins. When binding anti-apoptotic Bcl-2 proteins, BH3-only proteins are proposed to occupy the hydrophobic groove and neutralize their anti-apoptotic function (182). The interactions between members of the BH3-only protein family and anti-apoptotic Bcl-2 proteins are highly variable. For example, BH3 peptides from Bim and PUMA strongly interact with all anti-apoptotic Bcl-2 proteins whereas Noxa binds strongly only to Mcl-1 and A1 (46). Select BH3-only proteins including tBid, Bim, and PUMA are proposed to activate pro-apoptotic multi-domain Bcl-2 proteins by a direct and yet temporary interaction (43, 83, 164, 169). Studies undertaken with Bax has identified a unique interaction interface between multi-domain pro-apoptotic Bcl-2 protein and the BH3-ony proteins PUMA and tBid (43). The hydrophobic face of the amphipathic α-helix BH3 region in PUMA and tBid is still necessary for their interaction with Bax (43). However, instead of occupying the hydrophobic groove of the Bax, PUMA and tBid interact with the first amphipathic α-helix within the N-terminal region of Bax (43). Whether or not a similar mechanism exists for the interaction of Bak with BH3-only proteins is not yet clear.

1.5.3.3 Multidomain proapoptotic Bax and Bak

Gene deletion studies have played an invaluable role in understanding the function of various members of the Bcl-2 during apoptosis. An important discovery of how apoptosis is regulated at the mitochondria came to light by generating mice deficient in both Bax and Bak. While inactivation of either Bak or Bax alone had little impact in the development and phenotype of mice, mice deficient in both Bak and Bax died perinatally with few surviving to adulthood (180, 355). The few surviving mice showed severe developmental and phenotypic abnormalities as a result of decreased levels of apoptosis (180). In vitro, cells doubly deficient in Bak and Bax are resistant to numerous death stimuli, attributable to the lack of cytochrome c release, indicating a dominant role for both proteins in regulating apoptosis (68, 180, 355). It is now clear that Bak and Bax are obligate gatekeepers responsible for outer mitochondrial membrane permeabilization during apoptosis.

Both Bak and Bax are tail-anchored proteins but their subcellular localization differs significantly. The inactive version of Bak is constitutively localized to the outer mitochondrial membrane whereas inactive Bax is located predominantly in the cytoplasm or loosely associated with intracellular membranes (116, 363). The hallmark feature of Bak and Bax activation is a multi-step conformational rearrangement culminating in their homo-oligomerization in the outer mitochondrial membrane that coincides with the permeabilization of the outer mitochondrial membrane and the release of cytochrome c (9, 10, 354).

The insertion of Bax into mitochondria relies on its C-terminal transmembrane region. The solution structure of Bax reveals that the C-terminal transmembrane region is folded back into the hydrophobic pocket formed by the BH1, BH2, and BH3 domains (Figure 1-10B) (314). The insertion of Bax into mitochondria therefore, requires a dramatic structural reorganization. During apoptosis, Bax undergoes a conformational change in which the N-terminus becomes exposed and the C-terminal transmembrane region is liberated from the hydrophobic pocket (10, 42, 278). The conformational changes of Bax are under the control of two internal prolines that act as molecular switches (42, 278). Proline 13, which is part of the first α-helix, and proline 168 which lies just upstream of the C-terminal transmembrane region, both mediate the initial N-terminal exposure of Bax that is necessary but not sufficient to trigger insertion (42, 278). The insertion of Bax into the outer mitochondrial membrane is under the control of proline 168 which is required to unleash the C-terminal transmembrane region from the hydrophobic pocket (42, 278). How both proline residues regulate Bax activation is unclear. It has been proposed that modulation of Bax, in particular the two prolines, may be regulated by post-translational modifications or direct interactions with regulatory proteins (42, 278). Indeed anti-apoptotic cellular proteins including Ku70, ε14-3-3 and humanin have been found to interact with the N-terminal region of Bax (119, 229, 273). This data supports a model where anti-apoptotic proteins lock Bax in its inactive globular form and must be displaced in order for Bax to become active. This model is further supported by recent evidence that identified the first N-terminal α-helix domain in Bax as the interaction motif for PUMA and tBid (43). The interaction between the first N-terminal α-helix in Bax and the amphipathic BH3 region of PUMA or tBid was necessary for conformational restructuring and activation of Bax (43).

Similar to Bax, Bak is constitutively expressed in cells as an inactive protein. Bak however, is found constitutively localized to the outer mitochondrial membrane and is thought to be held in its inactive state via interactions with cellular proteins (48, 170). Bak activation involves restructuring of the protein which is initiated by exposure of the N-terminus and subsequent homo-oligomerization (115, 116, 354, 371). Recently the anti-apoptotic Bcl-2 protein, Mcl-1, was found to constitutively interact with Bak to inhibit its activation (60, 170, 362). In a manner analogous to its interaction with BH3-only proteins, the hydrophobic pocket of Mcl-1 incorporates the BH3 domain of Bak (170). Evidence indicates that Mcl-1 may function as a dominant inhibitor of Bak activation which must be removed for apoptosis to occur. Indeed, Mcl-1 itself is removed/lost during numerous apoptotic cascades either by proteosomal degradation, caspase cleavage or granzyme B cleavage (8, 60, 121, 170, 227, 356). Other data suggest that non-Bcl-2 pro-apoptotic proteins such as p53 directly liberate Bak from Mcl-1 (170). Furthermore, BH3-only proteins may trigger Bak activation by interacting with Mcl-1 or Bak to disrupt the Mcl-1-Bak complex (36, 46, 290, 291). In addition to interacting with Mcl-1, the inactive conformer of Bak also constitutively interacts with VDAC2, a low abundance isoform of VDAC (48). Following an apoptotic stimulus, VDAC2 inhibits Bak activation by preventing exposure of the N-terminus of Bak and subsequent homo-oligomerization (48). VDAC2 binds to the hydrophobic pocket of Bak and can be displaced by the BH3-only proteins Bad, Bim, and tBid (48). The physiological importance of VDAC2 in regulating Bak is unclear since VDAC2 is expressed in low abundance.

1.5.3.4 Anti-apoptotic Bcl-2 proteins

In simple terms, the pro-survival Bcl-2 proteins prevent apoptosis by inhibiting the release of cytochrome c. How anti-apoptotic Bcl-2 proteins inhibit cytochrome c release and whether all anti-apoptotic Bcl-2 proteins display mechanistic redundancy are two prevailing questions that continue to be the focus of much research. A number of competing pro-survival models have been postulated for anti-apoptotic Bcl-2 proteins. One model proposes that anti-apoptotic proteins regulate the PTP (35, 377). The exact composition of the PTP is controversial however, the essential components of the PTP are thought to include the outer membrane proteins VDAC, the peripheral benzodiazepine receptor (PBR), the inner membrane protein ANT and the matrix protein cyclophilin D (57, 345). Bcl-XL has been reported to interact directly with VDAC, while Bcl-2 has been reported to interact with the adenine nucleotide translocator (ANT) (36, 290, 291). Another model suggests anti-apoptotic Bcl-2 proteins are functionally dominant to pro-apoptotic Bcl-2 family proteins and serve to block Bak/Bax activation. Evidence indicates anti-apoptotic Bcl-2 proteins directly interact with Bak and Bax to inhibit their oligomerization (48, 266). Alternatively, anti-apoptotic Bcl-2 proteins have a strong binding affinity for BH3-only proteins suggesting their function is to sequester BH3-only proteins to prevent the activation of Bak and Bax (46, 49, 135). Each anti-apoptotic Bcl-2 protein is proposed to have distinct biological functions. In support of this, each anti-apoptotic Bcl-2 protein displays a selective binding profile to members of the BH3-only proteins (46). Furthermore, genetic inactivation of individual anti-apoptotic proteins reveals diverse phenotypes despite overlapping anti-apoptotic Bcl-2 expression profiles (257). Does the distinct biological function of anti-apoptotic Bcl-2 proteins indicate differential strategies to inhibit apoptosis? Indeed, certain anti-apoptotic Bcl-2 proteins differentially interact and regulate Bak oligomerization. As mentioned in the previous section, Mcl-1 constitutively interacts with Bak to inhibit Bak activation whereas Bcl-2 preferentially interacts with active Bak to inhibit Bak oligomerization (60, 135, 170, 266). Furthermore, Mcl-1 is proposed to have a unique apical role and Bcl-XL can not functionally replace Mcl-1 (227). Overall, although the anti-apoptotic Bcl-2 proteins all function to inhibit apoptosis, each protein likely has a specific biological role and may exercise slightly different mechanisms to inhibit apoptosis.

1.5.4 Mechanisms of Cytochrome c Release

The mechanism by which pro-apoptotic proteins are released from mitochondria is a topic of intense debate and scrutiny. Three mechanisms have been proposed to account for the release of pro-apoptotic proteins from the intermembrane space (Figure 1-12 and -13). The first mechanism involves the inner membrane PT, which results in matrix swelling and rupture of the outer membrane (202). The second mechanism involves the formation of pores in the outer membrane that act as molecular doorways for pro-apoptotic intermembrane proteins to enter the cytoplasm (202). The third mechanism amalgamates features of the first two models and involves remodelling of the mitochondria (284). The majority of studies that have analyzed the mechanism of mitochondrial protein release during apoptosis have focused on the release of cytochrome c however, the release of specific intermembrane proteins may differ from cytochrome c release.

1.5.4.1 The mitochondrial permeability transition model

The inner mitochondrial membrane is predominantly an impermeable membrane. Under some conditions, the inner membrane permeability increases to allow passage of water and molecules up to 1500 Daltons and is referred to as PT (113, 162). The PT is mediated by opening of the PTP that is triggered by various pathophysiological conditions including Ca^{+2} influx, depolarization of the inner membrane, oxidizing agents and metabolic molecules such as fatty acids (377). Sustained opening of the PTP accompanies irreversible loss of the inner mitochondrial membrane potential, blockage of ATP synthesis, Ca^{+2} efflux into the mitochondrial matrix, generation of superoxides, and depletion of NADH collectively resulting in the impairment of mitochondrial function (160). Swelling of the matrix is a secondary consequence of prolonged PT. Since the inner membrane surface area far exceeds the outer membrane surface area, swelling of the matrix leads to nonselective rupture of the outer membrane and the release of intermembrane proteins including cytochrome c (Figure 1-12A) (161, 238).

Although this mechanism represents one mode of cytochrome c release, several observations suggest that prolonged PT has very limited, if any, role in apoptosis. One significant drawback to the PT model is the apparent lack of matrix swelling and herniation of the outer membrane during apoptosis induced under physiological conditions (82, 157, 290, 292). The most damaging data against this model however, has been achieved with mice deficient in cyclophilin D (15, 221). These mice develop normally with no developmental defects attributed to the inhibition of apoptosis. Furthermore, cyclophilin D deficient mice undergo apoptosis induced by a number of

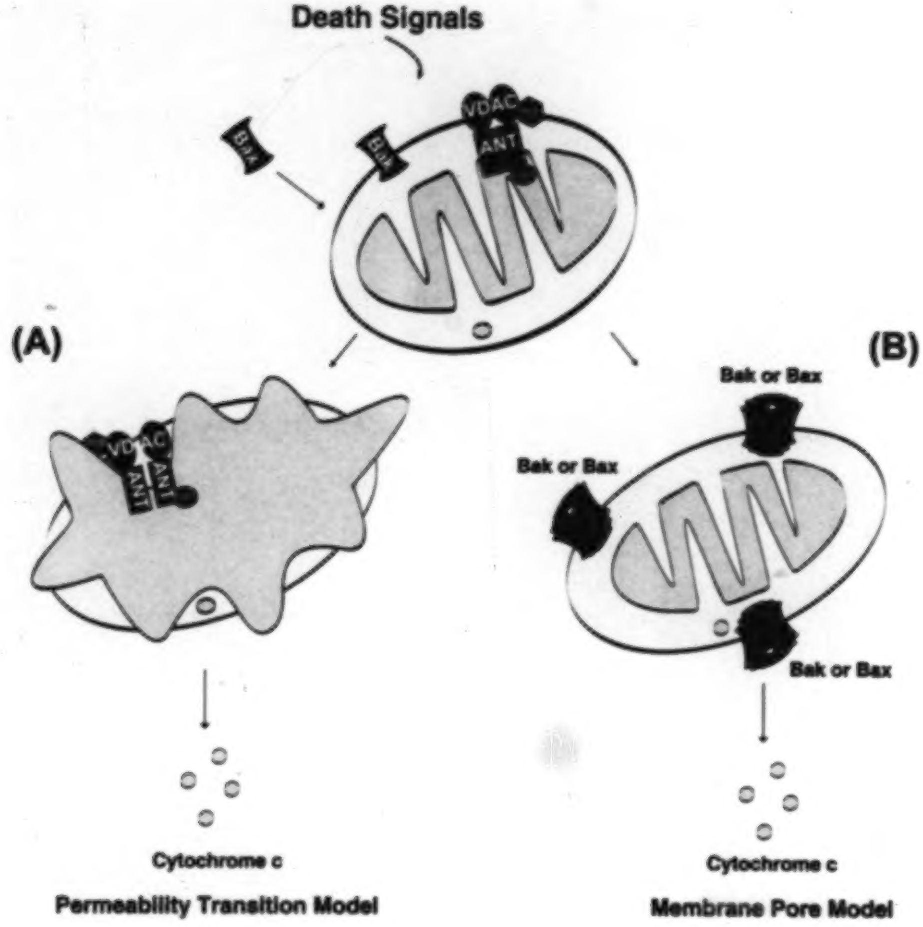

Death Signals
Bak
Bax
VDAC
ANT
(A)
(B)
Bak or Bax
Bak or Bax
Bak or Bax
Cytochrome c
Permeability Transition Model
Cytochrome c
Membrane Pore Model

Figure 1-12 Classical models of cytochrome c release.
(A) The permeability transition model proposes that the release of cytochrome c is a result of prolonged opening of the permeability transition pore (PTP) complex resulting in matrix swelling and rupture of the outer membrane. (B) The membrane pore model proposes that cytochrome c is released as a result of direct permeabilization of the outer membrane by the formation of pores that are dependent on Bak or Bax homo-oligomerization. The PTP is composed of voltage dependent anion channel (VDAC), adenine nucleotide translocator (ANT), peripheral benzodiazepine receptor (PBR), and cyclophilin D (CyD).

stimuli including the expression of BH3-only proteins and Bax (15, 221). Interestingly, the absence of cyclophilin D prevents cells from undergoing necrotic cell death in response to reactive oxygen species and Ca^{+2} supporting a significant role for cyclophilin D, and the PTP, in necrosis (15, 221). Overall, this data suggests that the current PT model of cytochrome c release likely reflects a necrotic cell death more than an apoptotic cell death.

1.5.4.2 The outer membrane pore model

The absence of mitochondrial swelling during apoptosis suggests that cytochrome c and other intermembrane space proteins can be released without gross perturbations of the outer membrane as one might expect by the generation of membrane pores (Figure 1-12B). Numerous models have been proposed to account for this phenomenon with a clear emphasis on the role of Bak and Bax (161). Bak and Bax are essential for outer mitochondrial membrane permeabilization during apoptosis and therefore each mechanism of membrane permeabilization must include these two pro-apoptotic proteins (180, 355). Current models suggest that Bak and Bax form large lipid channels in the outer membrane, interact with pre-existing mitochondrial channels such as the PTP, form exclusive pores, or globally disrupt the outer mitochondrial membrane by regulating the mitochondrial fission and fusion machinery (9, 151, 165, 203, 291). Whatever the mechanism(s) of outer membrane permeabilization, Bak and Bax oligomerization is a critical step in liberating cytochrome c from the mitochondria during apoptosis (9, 354).

1.5.4.3 The mitochondrial remodelling model

Although the role of prolonged PT during apoptosis has likely been excluded as the mechanism by which cytochrome c is released, a number of observations support a role for the PTP in apoptosis. Mice that overexpress cyclophilin D display elevated levels of apoptosis in cardiac cells when compared to wild-type mice (15). Lymphocytes treated with protoporphyrin IX, an activating ligand for the PBR, undergo DNA fragmentation that is inhibited by pretreating cells with the broad spectrum caspase inhibitor zVAD.fmk or by the addition of bongkrekate, a specific ANT ligand that prevents opening of the PTP (198). Members of the Bcl-2 family including Bak, Bax, Bcl-2 and Bcl-XL interact and modulate PTP activity as do several viral and bacterial encoded apoptotic regulators (33, 34, 48, 82, 83, 142, 203, 251, 289-292). Evidence indicates that transient opening of the PTP, and therefore transient PT that does not result in matrix swelling occurs under conditions that coincide with cytochrome c release leading to apoptosis (251, 283). Collectively, a new model has been put forth to accommodate the role of the PTP and outer membrane pore formation in cytochrome c release during apoptosis (283).

The majority of cytochrome c (~85%) is located in the mitochondrial cristae which is the major site of oxidative phosphorylation (107, 201). The release of cytochrome c however is kinetically rapid and complete suggesting that remodelling of the cristae is necessary to liberate intracristae cytochrome c to a site that allows access to the outer membrane (107, 283). Using biochemical and high-voltage electron microscopic tomography, Korsmeyer and colleagues provide a novel mechanism that

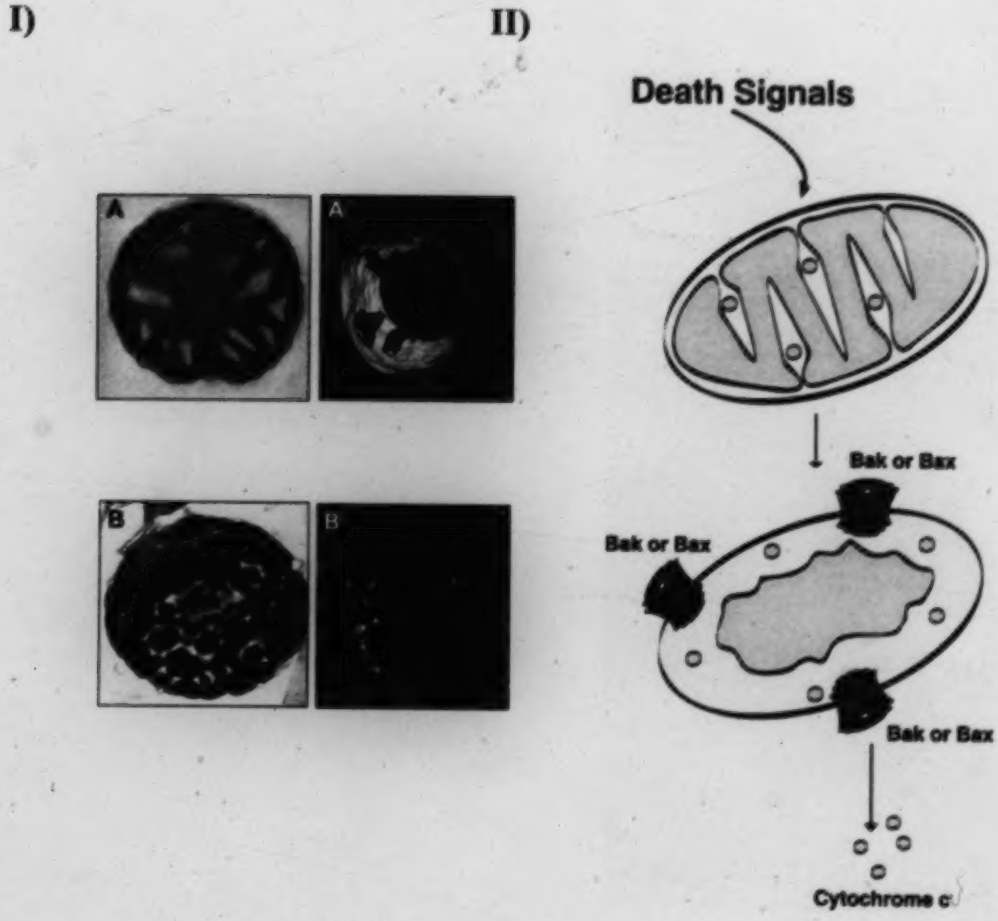


Figure 1-13 Mitochondrial cristae remodelling and cytochrome c release. (I) Electron microscopy tomography of mitochondria. Electron microscopy of healthy mitochondria (A) or mitochondria undergoing cristae remodelling (B). Surface rendered tomographic reconstructions of healthy mitochondria (A') or mitochondria undergoing remodelling (B'). The outer membrane is shown in red, the inner membrane is depicted in yellow and cristae are green. (II) Schematic representation of the mitochondrial remodelling model for the release of cytochrome c. Following an apoptotic stimulus, the inner membrane undergoes ultrastructural changes including the expansion and eventual loss of cristae junctions that is proposed to liberate the majority of cytochrome c. Bak and Bax undergo conformational changes leading to their homo-oligomerization that is required for the release of cytochrome c out of the intermembrane space. Adapted from Scorrano, L. *et al.* 2002 (283).

accounts for both the need for outer membrane pore formation and PTP opening (Figure 1-13) (283). The authors found that mitochondria treated with tBid underwent ultrastructural changes in which the mitochondrial matrix compressed, the intermembrane space expanded, the cristae junctions and the cristae disappeared that coincided with the release of cytochrome c. The authors suggested that cytochrome c release was a two step process that was initiated by remodelling of the inner mitochondrial membrane to liberate intra-cristae cytochrome c stores followed by passage through the outer membrane via Bak homo-oligomers. Interestingly, Bak activation and inner membrane remodelling were separate and exclusive events: the addition of cyclosporine A blocked cristae remodelling but not Bak oligomerization without the release of cytochrome c. In addition, the absence of Bak inhibited cytochrome c release even when mitochondrial cristae were remodelled. Similar observations of cristae remodelling during apoptosis were obtained by Thompson and colleagues who found that membrane uncouplers induced remodelling of the cristae that was necessary for Bak dependent release of cytochrome c (110). Furthermore, Shore and colleagues observed cristae remodelling during Bik induced cytochrome c release mediated by mitochondrial Ca^{+2} uptake and Drp1 localization to the mitochondria(102).

1.6 Viral Anti-apoptotic Strategies

Specialized immune effector cells play a key role in removing virus infected cells by inducing apoptosis (18, 268). In addition, virus infection alone activates an intrinsic innate response in which the infected cell undergoes apoptosis (86, 265). As a result, viruses have developed mechanisms to inhibit apoptosis (Figure 1-14).

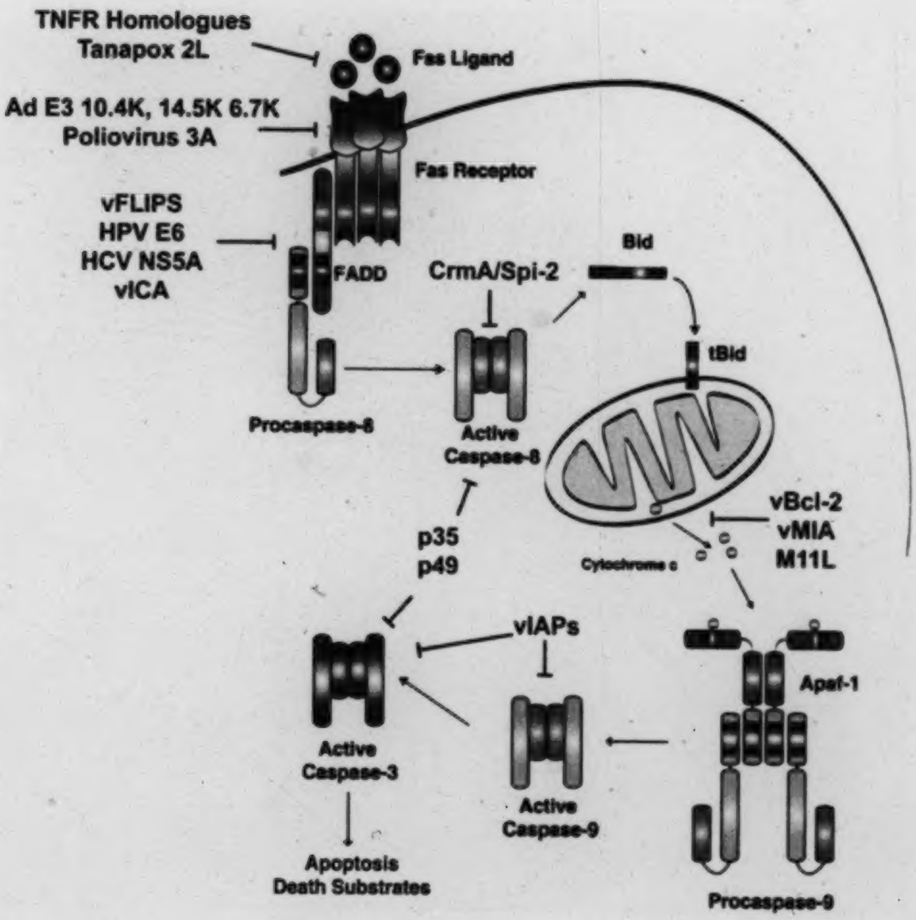

TNFR Homologues
Tanapox 2L
Fas Ligand
Ad E3 10.4K, 14.5K 6.7K
Poliovirus 3A
Fas Receptor
vFLIPS
HPV E6
HCV NS5A
vICA
FADD
CrmA/Spi-2
Bid
tBid
Procaspase-8
Active Caspase-8
vBcl-2
vMIA
M11L
p35
p49
Cytochrome c
vIAPs
Apaf-1
Active Caspase-3
Active Caspase-9
Apoptosis Death Substrates
Procaspase-9

Figure 1-14 Viral inhibitors of apoptosis.
Viruses have evolved a number of mechanisms to inhibit apoptosis. This includes interfering with death receptor activation, interference of DISC formation, caspase inhibition, and regulating the mitochondria. Viruses encode cellular homologues such as TNFR decoy receptors, vFLIPS, vIAP and vBcl-2 proteins to inhibit apoptosis. In addition, a number of viral encoded proteins with no homology to known anti-apoptotic proteins exist.

1.6.1 Inhibition of Death Receptor Mediated Apoptosis

Immune effector cells induce apoptosis of virally infected cells by secreting cytokines such as TNFα or through direct contact of virus infected cells via FasL (12, 41, 45, 353). The binding of TNFα or FasL to their cognate death receptors initiates a distinct series of events aimed at terminating viral replication by inducing cell death (12). The lethal effect of death receptor activation indicates that it would be an advantage for viruses to usurp this apoptotic process.

Members of the poxvirus family have evolved a unique strategy to intercept TNFα-TNFR interactions. Poxviruses secrete viral encoded TNFR homologs that bind to and block the effector function of TNFα (20). Viral TNFRs display sequence similarity to the extracellular ligand binding domain of TNFR but lack transmembrane and intracellular signalling domains. The first TNFR homologs to be described were the T2 proteins encoded by Shope fibroma virus (S-T2) and myxoma virus (M-T2) (299, 300, 332). Follow up studies with M-T2 indicated that the protein is secreted as either a monomer or dimer, each with the capability to bind rabbit TNFα but not murine or human TNFα (281, 282). Deletion of the M-T2 gene in myxoma significantly attenuates the virus in vivo suggesting an important role in virus virulence (332). Cowpox virus encodes four distinct TNFR homologs including CrmB, CrmC, CrmD, and CrmE each with differential expression patterns, ligand specificities and cytokine neutralization capabilities (136, 187, 271, 301). Examples of secreted viral TNFR are present in the majority of Orthopoxviruses but most strains of vaccinia virus are devoid of viral TNFR homologs due to frameshift or nonsense mutations (5, 261).

Sequence analysis of Yatapoxviruses, another genera of poxviruses, indicate these viruses do not encode any obvious TNFR homologs (38, 167). One member of the Yatapoxvirus genus, Tanapox, exhibits TNF-binding activity attributed to the 2L protein (39, 239). As with the viral TNF receptor homologs, 2L is secreted, displays specificity for its ligand and protects cells from TNF induced cytolysis by inhibiting TNFα binding to TNFR receptors (39).

Rather than encode decoy receptors, adenoviruses (AD) encode proteins that downregulate death receptors from the cell surface as a means to inhibit apoptosis. Early during infection, adenoviruses express E3 10.4K and E3 14.5K proteins which heterotrimerize at a 2:1 ratio to form the receptor internalization and degradation (RID) complex (307). The RID complex localizes to the surface membrane and participates in the downregulation of cell surface levels of Fas (81, 295, 323), TRAIL receptor 1(24, 324), TNFR1 (93), and with the help of E3 6.7K, TRAIL receptor 2 (178). Through a mechanism involving a dileucine sorting motif in E3 10.4K and a tyrosine sorting motif in E3 14.5K, the RID complex mediates the internalization of death receptors from the cell surface into the endosomal pathway where the receptors are ultimately degraded in lysosomes (130, 179, 370). Receptor downregulation is another mechanism viruses use to modulate apoptosis. The poliovirus 3A protein downregulates the surface levels of TNFR1 by disrupting ER-Golgi trafficking and human papillomavirus E5 protein reduces surface levels of Fas by an unknown mechanism (148, 224). Human cytomegalovirus (HCMV) also eliminates TNFR1 from the cell surface however the gene(s) responsible have not been identified (14).

1.6.2 Interference of DISC Formation and Function

The formation of the death inducing signalling complex is essential for death receptor mediated apoptosis (245). Ligand binding to death receptors triggers the assembly of the DISC that is necessary for caspase-8 activation. Human papillomavirus 16 (HPV) E6 protein prevents TNF and FasL induced apoptosis by two unique mechanisms. HPV E6 protein interacts with the DD region of TNFR1 to block the recruitment of TRADD and also physically associates with the DED motif of FADD to induce FADD's degradation (94, 95). The inhibition of TNFα- but not Fas-induced apoptosis by the hepatitis C virus NS5A protein has also been reported (194, 215, 236). The NS5A protein physically associates with TRADD and prevents the association between TRADD and FADD to short-circuit the apoptotic cascade (194, 236).

Recruitment of procaspase-8 to the death inducing signalling complex is a pivotal step in the death receptor cascade. Several viruses such as the γ-herpesviruses, encode viral FLICE (Fas-associated death-domain-like interleukin-1β converting enzyme)-inhibitory proteins (vFLIPs) which consist of two DEDs and interact with FADD and/or procaspase-8 through homotypic interactions to prevent apoptosis in a dominant negative manner (27, 137, 350). The poxvirus molluscum contagiosum encodes two vFLIPs, MC159 and MC160, both of which interact with FADD and procaspase-8 via DED-DED interactions (27, 101, 137, 296, 320, 327). However the ability of the MCV vFLIPs to bind FADD or procaspase-8 is not the sole determinant of their anti-apoptotic function. While still maintaining its capability to bind FADD and pro-caspase-8, MC160 displays very little if any anti-apoptotic activity likely as a result of its degradation during death receptor mediated apoptosis (296). In addition, mutagenesis studies with MC159 indicate that its interaction with either FADD or procaspase-8 is not sufficient for anti-apoptotic inhibition (101). Thus the anti-apoptotic ability of the MCV vFLIPs requires an unknown function that is necessary to inhibit apoptosis. One possible explanation involves MC159 facilitating NFκB activation that is associated with induction of anti-apoptotic pathways (348)

The betaherpesvirus HCMV encodes UL36, the founding member of a novel class of anti-apoptotic proteins that share no homology to vFLIPs yet regulate procaspase-8 activation (207). Similar to vFLIPs, the viral inhibitor of caspase-8 activation (vICA) functions to inhibit caspase-8 activation by binding to and inhibiting the activation of procaspase-8 however, whether the inhibition is a result of inhibiting recruitment of caspase-8 to the DISC or blocking caspase-8 homodimerization is unclear. vICA exhibits specificity to bind to the prodomain of procaspase-8. Whether vICA binds to the DED domain in the procaspase-8 is not known but its inability to bind FADD suggests that vICA may interact with procaspase-8 independent of the DED motifs (297). Other betaherpesviruses sequenced to date encode conserved vICA homologs (207). Of those tested thus far, all exhibit anti-apoptotic function in response to Fas suggesting the conservation of vICA function among betaherpesviruses (207).

1.6.3 Viral Encoded Caspase Inhibitors

Baculoviruses and poxviruses express proteins that directly inhibit apoptosis by targeting active caspases. Baculovirus encode two caspase inhibitors referred to as p35 and p49. The p35 protein inhibits most caspases but noticeably, not the apical drosophiha caspase DRONC (40, 365, 372). P35 acts as a pseudosubstrate for active caspases that once cleaved, remains covalently associated with the caspase to block caspase active site (40, 124, 211, 372). The p35 homolog, p49 exhibits a broad caspase inhibitory activity, is cleaved by caspases and forms a covalent complex with the protease (144). In contrast to p35, p49 inhibits DRONC suggesting an apical role for the caspase inhibitor (144, 376). An additional 120 amino acid insertion and a distinct tetrapeptide cleavage site are predicted to influence the target specificity and function of p49 and may account for its ability to inhibit DRONC (376).

The cowpox encoded cytokine response modifier A (CrmA) is a member of the serine protease inhibitor (serpin) protein family (250, 304, 373). Serpins function by acting as pseudosubstrates that form stable inhibitory complexes with their target proteases (104). In the case of CrmA, the pseudosubstrate region of the protein is found within its reactive site loop (158, 373). CrmA is a strong inhibitor of caspase-1, the caspase that converts pro-IL1-β to its active form (260). CrmA expression also protects cells from a number of apoptotic stimuli attributed to its ability to interact with caspase-8 and granzyme B (255, 260, 373). Other poxviruses, in particular most members of the Orthopoxvirus genera, including ectromelia virus, rabbitpox virus, variola virus and certain strains of vaccinia virus encode CrmA homologs, referred to as serine protease inhibitor – 2 (Spi-2). Spi-2 proteins are highly conserved within the reactive site loop and of those tested, are functionally similar to CrmA (73, 154, 190, 331).

Members of the leporipoxvirus genera encode Serp-2, a serine protease inhibitor with 35% sequence identity to CrmA/Spi-2 (246). Within the reactive site loop, Serp2 is 36% identical and 62% similar to CrmA suggesting that it may function as a caspase inhibitor. When tested *in* vitro, the myxoma virus Serp-2 is capable of inhibiting granzyme B and pro-IL1-β, albeit at a considerably lower rate compared to CrmA/Spi-2, but is not able to inhibit caspase-8 activity (330). Whether Serp-2 is a bonafide anti-apoptotic protein remains uncertain. Serp-2 expression is unable to rescue a CrmA deleted cowpox virus from virus-induced apoptosis suggesting it does not function as an anti-apoptotic protein under these conditions (330). Although the ability of Serp-2 to inhibit apoptosis remains uncertain, the Serp-2 knockout virus exhibits attenuated disease symptoms and elevated levels of lymphocytes in draining lymphnodes during infection of rabbits indicating Serp-2 is important for myxoma virus virulence (213).

1.6.4 Viral IAPs

In drosophila, caspase activation is regulated by IBM proteins Grim/Hid/Reaper (26). Upon an apoptotic stimulus, IBM proteins are activated allowing them to interact with cellular IAP proteins thereby liberating active caspases (26). Insect IAPs encoded by members of the *Baculoviridae* and *Entomovirinae* are highly effective at inhibiting apoptosis but utilize a different mechanism in comparison to cellular IAPs (28, 58, 176, 177). The prototypic baculovirus IAP from *Orgyia pseudotsugata*, Op-IAP, is incapable of inhibiting drosophila caspase activation *in vitro*, yet functions as a strong inhibitor of apoptosis in cells suggesting that Op-IAP functions upstream of caspase activation (364). Compelling evidence provides a model in which Op-IAP inhibits apoptosis by binding and neutralizing the drosophila IBM proteins Hid/Grim/Reaper (347). In support of this, Op-IAP also inhibits apoptosis in mammalian cells while being unable to inhibit mammalian caspase activation *in vitro* (361). Op-IAP sequesters cytoplasmic SMAC/Diablo, an IBM binding protein, preventing its function as a liberator of caspases from cellular IAP inhibition (361). Similar results have recently been observed with the poxvirus AmEPV (Amsacta moorei entomopoxvirus) IAP protein which preferentially bind the IBM tetrapeptide motifs from Grim/Hid/Reaper to prevent apoptosis (177).

African swine fever virus (ASFV) also encodes an IAP that prevents apoptosis when expressed ectopically or during virus infection (228). Unlike the insect virus IAPs however, ASFV IAP interacts with activated caspase-3 suggesting that this IAP has adopted a mechanism similar to cellular IAPs (228). Taken together, these results highlight how viral IAPs have evolved to inhibit caspase activation by different mechanisms.

1.6.5 Virus-encoded Proteins that Regulate the Mitochondria

Many viruses have taken advantage of the important regulatory role mitochondria serve during apoptosis by encoding anti-apoptotic proteins that localize to mitochondria. A number of viral encoded proteins inhibit multiple apoptotic cascades simply by regulating the mitochondrial checkpoint. These virally encoded inhibitors are classified depending on their homology to cellular Bcl-2 family proteins as either viral Bcl-2-like or viral non-Bcl-2-like proteins.

1.6.5.1 Viral Bcl-2-like proteins

Virus encoded Bcl-2 homologs have been identified in many viruses. All gamma herpesviruses sequenced thus far, the alpha herpesviruses Marek's disease like virus, African swine fever virus, adenovirus and fowlpox virus all encode viral Bcl-2 (vBcl-2) homologs (61, 122). Viral Bcl-2 proteins share little homology at the primary amino acid sequence to their cellular counterparts but contain conserved motifs that are important for their anti-apoptotic function (61, 122). Viral Bcl-2 proteins contain both a BH1 and BH2 with the exception of γHV68 Bcl-2 and E1B 19K which lack the BH2 domain (122). Sequence analysis indicates that the BH3 and BH4 domains are poorly conserved among vBcl-2 proteins suggesting these domains are not essential to inhibit apoptosis or alternatively, they have been modified for the advantage of the virus. Similar to their cellular counterparts, vBcl-2 proteins are predicted to contain eight α-helices and a putative transmembrane region suggesting structural conservation even in the absence of BH3 and BH4 domains (122). With the exception of BALF1 encoded by Epstein-Barr virus, all vBcl-2 proteins tested thus far are functional homologs of their anti-apoptotic relatives (22, 61). Whether all vBcl-2 proteins utilize the same mechanism to inhibit apoptosis however, has yet to be determined.

The Kaposi's sarcoma herpesvirus Bcl-2 (KS-Bcl-2) protein was the first vBcl-2 structure to be determined (139). The overall structure of KS-Bcl-2 is similar to other cellular anti-apoptotic Bcl-2 family members including a central α-helix hairpin, α5 and α6, that are surrounded by six amphipathic α-helices. In addition, the hydrophobic groove is conserved in the viral protein although the specificity for binding BH3 peptides is different from that observed with Bcl-2 and Bcl-XL (139). Cellular Bcl-2 and Bcl-XL both have a strong affinity for the BH3 peptide of Bad when compared to Bak and Bax BH3 peptides whereas KS-Bcl-2 exhibits a stronger affinity for Bak and Bax BH3 peptides in vitro (139). This suggests a model where KS-Bcl-2 preferentially binds to Bak and Bax to inhibit apoptosis. Whether this mechanism is correct is unclear since a direct interaction between KS-Bcl-2 and full-length Bak or Bax has not been detected (47).

The solution structure of the vBcl-2 homolog BHRF1 encoded by Epstein-Barr virus has also been solved. The overall structure of BHRF1 is similar to other Bcl-2 family proteins except it lacks the hydrophobic binding groove (140). Consistent with the absence of the hydrophobic cleft, BHRF1 is incapable of binding BH3 peptides derived from Bak, Bax, Bik and Bad although a direct interaction between full-length Bak but not Bax has been detected (140, 223, 319). Collectively, this suggests that BHRF1 interacts in a BH3-independent manner to Bak. Furthermore, it is interesting to speculate that BHRF1 may have an anti-apoptotic mechanism independent of its ability to bind BH3-domains present in pro-apoptotic Bcl-2 proteins.

One of the best studied vBcl-2 is the E1B 19K protein encoded by adenovirus and is a strong inhibitor of apoptosis induced by numerous stimuli including adenovirus infection and TNFα treatment (61). When expressed during virus infection, E1B 19K preferentially interacts and inhibits the activity of Bak and Bax (59, 60, 242, 310, 311). The interaction between E1B 19K and Bak or Bax is dependent on the conformational activation of Bak and Bax in which the N-terminus is exposed (59, 242, 310, 311). In both cases the oligomerization of Bak and Bax is prevented by E1B 19K to preserve the integrity of the mitochondria.

To date, members of the avipoxvirus genera, including fowlpox and canarypox, are the only known poxviruses to encode vBcl-2 proteins (2, 329). Similar to other vBcl-2 proteins, FPV039, encoded by fowlpox virus and CNPV058, encoded by canarypox virus, contain conserved BH1 and BH2 domains as well as a putative C-terminal transmembrane domain suggesting these proteins have the capacity to inhibit apoptosis. Indeed, recent work in our lab indicates that FPV039 inhibits apoptosis. In the absence of infection, FPV039 predominantly localizes to the mitochondria and inhibits mitochondrial mediated apoptosis induced by TNFα (L. Banadyga and M. Barry unpublished data). Furthermore, biochemical studies have identified a clear interaction between FPV039 and Bak as well as Bax suggesting that FPV039 functions to inhibit apoptosis by regulating these proteins. Whether or not FPV039 preferentially interacts with active or inactive conformations of Bak and Bax is currently being tested.

1.6.5.2 Viral non-Bcl-2 like proteins

The expression vBcl-2 proteins is a classical example of how viruses hijack cellular proteins for their use. A number of viruses however, regulate the mitochondria during apoptosis while lacking obvious cellular Bcl-2 homologs. As a result, a number of non-Bcl-2-like viral proteins that function at the mitochondria to inhibit apoptosis have been discovered.

During the lytic stage of its life cycle, Kaposi's sarcoma herpesvirus (KSHV) expresses vBcl-2 and K7, two anti-apoptotic proteins that localize to the mitochondria. The K7 protein is structurally related to a spliced version of human survivin, survivin-ΔEx3(351). K7 is a molecular hybrid that contains two anti-apoptotic motifs including the N-terminal portion of a BIR domain and a putative BH2 domain separated by a central hydrophobic region (351). Evidence indicates that K7 encompasses multiple strategies to inhibit apoptosis depending on the particular inducer. K7 interacts with activated caspase-3 and cellular Bcl-2 through its BIR and BH2 motifs respectively to inhibit apoptosis induced by death receptors or the overexpression of Bax (351). In addition, K7 interacts with cellular calcium-modulating cyclophilin ligand (CAML), a protein that regulates intracellular calcium concentration (87). During apoptosis induced by thapsigargin, a SERCA inhibitor that induces transient elevation of cytosolic Ca^{+2}, K7 protects against apoptosis that is abolished by removing the CAML binding motif. K7 also interacts, via its central hydrophobic region, with the protein-linking integrin-associated protein and cytoskeleton 1 (PLIC1), a protein that prevents p53 degradation by the proteosome (88, 156). During apoptosis triggered by etoposide, K7 is thought to antagonize PLIC1 activity resulting in enhanced degradation of p53 however whether this inhibition is attributable to PLIC1 function is not known (88). The fact that KSHV has evolved multiple mechanisms to regulate the mitochondrial checkpoint suggests this organelle is critical during virus infection.

Novel mitochondrial anti-apoptotic proteins have also been discovered in HCMV and myxoma virus, both of which lack open reading frames with homology to Bcl-2 family members. Viral mitochondrial inhibitor of apoptosis (vMIA) is an anti-apoptotic protein encoded by the first exon of the UL37 gene (UL37x1) in HCMV (106). Expression of vMIA inhibits apoptosis induced by a variety of stimuli including death receptor ligands, cytotoxic drugs and infection with HCMV lacking a functional vMIA (23, 106, 262, 346). Two domains within vMIA are necessary and sufficient for anti-apoptotic function; amino acids 5-34 which are necessary and sufficient for vMIA localization to the mitochondria and amino acids 118-147 which are necessary for vMIA anti-apoptotic function (125). Other primate CMVs contain functional vMIA homologs that are noticeably absent in rodent CMVs and other betaherpesviruses (207).

Studies aimed at understanding the mechanism of how vMIA inhibits apoptosis have identified its cellular interacting proteins. Initial studies indicated that vMIA interacts with the adenine nucleotide translocator but this binding activity does not appear to be required for vMIA anti-apoptotic function (11). Interestingly, how vMIA inhibits apoptosis may parallel the morphological changes associated with HCMV infection or ectopic expression of vMIA. Under healthy conditions, mitochondria form a highly dynamic reticular network (243). During apoptosis, mitochondria undergo fragmentation mediated by Drp1 and the formation of Bax oligomers at the site of scission (151, 243). The expression of vMIA, either ectopically or during virus infection, induces mitochondrial fragmentation in the absence of cell death attributable to the two identified functional domains that are required to inhibit apoptosis (208). One potential explanation for this unique morphology has recently been proposed. Work by two groups indicates that vMIA regulates Bax activation to inhibit apoptosis (11, 252). Surprisingly, expression of vMIA recruits Bax, either directly or indirectly, to mitochondria and leads to oligomerization of Bax (11, 252). This suggests that Bax is activated and is free to contribute to mitochondrial fission but somehow the expression of vMIA neutralizes the pro-apoptotic function of Bax. This data also suggests that Bax oligomerization confers a selective advantage to HCMV infection although this has not been addressed. Evidence in support of this hypothesis comes from studies involving murine cytomegalovirus (MCMV). Like HCMV, MCMV infection induces Bax to translocate to mitochondria and form higher order oligomers that bypasses exposure of its N-terminus yet prevents Bax dependant apoptosis (8). Thus, it is conceivable that the Bax oligomers identified during cytomegalovirus infection are distinct to those that mediate the release of proapoptotic proteins released from the mitochondria during apoptosis although this has yet to be tested.

With the exception of members of the avipoxvirus genera, no members of the poxvirus family encode obvious Bcl-2 homologues suggesting that poxviruses have evolved alternative mechanisms to regulate the mitochondrial apoptotic pathway. The myxoma-virus M11L protein is a recent addition to the family of anti-apoptotic non-Bcl-2-like mitochondrial proteins. Like other mitochondrial anti-apoptotic proteins, M11L maintains the mitochondrial integrity in the presence of death signals (84, 85, 349). M11L constitutively interacts with Bak and inhibits Bak-induced apoptosis but whether M11L inhibits Bak conformational changes and homo-oligomerization is not known (349). In addition to interacting with Bak, M11L directly associates with the PBR (85). Expression of M11L prevents both the loss of the mitochondrial membrane potential and release of cytochrome c induced by protoporphyrin IX, a PBR ligand (85). Whether this suggests a link between the PTP and Bcl-2 proteins or indicates that M11L has multiple strategies to regulate different proteins during apoptosis is not clear. Interestingly, the C-terminal transmembrane domain of M11L protects against loss of the inner mitochondrial membrane potential induced by protoporphyrin IX but not staurosporine which might suggest that M11L has specific domains that are required to protect against apoptosis induced by specific stimuli (85). Sequence analysis of viral genomes from poxvirus genera including *Suipoxvirinae*, *Capripoxvirnae* and *Yatapoxvirinae* all encode proteins with homology to M11L. Interestingly, members of the Orthopoxvirus genus are devoid of M11L-like proteins suggesting Orthopoxviruses may utilize a novel mechanism to regulate the mitochondrial checkpoint during apoptosis.

1.7 Thesis Objectives

The discovery of mitochondria as central regulators of apoptosis ushered in an exciting and fast-paced era of scientific research. The idea that viruses regulate mitochondria to inhibit apoptosis was revealed with the initial discovery of E1B 19K, a Bcl-2 homolog encoded by adenovirus (52, 357). With the sequencing of multiple viral genomes, other virus encoded Bcl-2 homologs were discovered (61, 122). In stark contrast a number of viruses did not contain open reading frames with homology to Bcl-2 (84, 106).

In 1990, the complete genome of vaccinia virus strain Copenhagen was sequenced (105). Analysis of the genome indicated that vaccinia virus did not contain any open reading frames with homology to Bcl-2. Despite the lack of a Bcl-2-like gene, the importance of the mitochondria led Michele Barry in the late 1990s to speculate that vaccinia virus encoded a unique anti-apoptotic protein that regulated the mitochondrial checkpoint and initial experimental data supported this hypothesis. This hypothesis served as a starting point for the research in this thesis.

In simple terms, this thesis is a collection of experiments, assembled into three sections, that has attempted to characterize how vaccinia virus regulates the mitochondria to inhibit apoptosis. These are outlined below.

Objectives:

i) Characterize the ability of vaccinia virus strain Copenhagen to modulate the mitochondrial apoptotic cascade.

ii) Identify the vaccinia virus open reading frame that regulates the mitochondrial checkpoint.

iii) Determine the mechanism of how F1L modulates apoptosis.

1.8 References

1. **Acehan, D., X. Jiang, D. G. Morgan, J. E. Heuser, X. Wang, and C. W. Akey.** 2002. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell **9:**423-32.
2. **Afonso, C. L., E. R. Tulman, Z. Lu, L. Zsak, G. F. Kutish, and D. L. Rock.** 2000. The genome of fowlpox virus. J Virol **74:**3815-31.
3. **Aggarwal, B. B.** 2003. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol **3:**745-56.
4. **Ahmad, M., S. M. Srinivasula, L. Wang, G. Litwack, T. Fernandes-Alnemri, and E. S. Alnemri.** 1997. Spodoptera frugiperda caspase-1, a novel insect death protease that cleaves the nuclear immunophilin FKBP46, is the target of the baculovirus antiapoptotic protein p35. J Biol Chem **272:**1421-4.
5. **Alcami, A., A. Khanna, N. L. Paul, and G. L. Smith.** 1999. Vaccinia virus strains Lister, USSR and Evans express soluble and cell-surface tumour necrosis factor receptors. J Gen Virol **80 (Pt 4):**949-59.
6. **Algeciras-Schimnich, A., L. Shen, B. C. Barnhart, A. E. Murmann, J. K. Burkhardt, and M. E. Peter.** 2002. Molecular ordering of the initial signaling events of CD95. Mol Cell Biol **22:**207-20.
7. **Alnemri, E. S., D. J. Livingston, D. W. Nicholson, G. Salvesen, N. A. Thornberry, W. W. Wong, and J. Yuan.** 1996. Human ICE/CED-3 protease nomenclature. Cell **87:**171.
8. **Andoniou, C. E., D. M. Andrews, M. Manzur, P. Ricciardi-Castagnoli, and M. A. Degli-Esposti.** 2004. A novel checkpoint in the Bcl-2-regulated apoptotic pathway revealed by murine cytomegalovirus infection of dendritic cells. J Cell Biol **166:**827-37.
9. **Antonsson, B., S. Montessuit, S. Lauper, R. Eskes, and J. C. Martinou.** 2000. Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. Biochem J **345 Pt 2:**271-8.
10. **Antonsson, B., S. Montessuit, B. Sanchez, and J. C. Martinou.** 2001. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem **276:**11615-23.
11. **Arnoult, D., L. M. Bartle, A. Skaletskaya, D. Poncet, N. Zamzami, P. U. Park, J. Sharpe, R. J. Youle, and V. S. Goldmacher.** 2004. Cytomegalovirus cell death suppressor vMIA blocks Bax- but not Bak-mediated apoptosis by binding and sequestering Bax at mitochondria. Proc Natl Acad Sci U S A **101:**7988-93.
12. **Ashkenazi, A., and V. M. Dixit.** 1998. Death receptors: signaling and modulation. Science **281:**1305-8.
13. **Atlas, R. M.** 1998. The threat of bioterrorism returns the fear of smallpox. Curr Opin Microbiol **1:**719-21.
14. **Baillie, J., D. A. Sahlender, and J. H. Sinclair.** 2003. Human cytomegalovirus infection inhibits tumor necrosis factor alpha (TNF-alpha) signaling by targeting the 55-kilodalton TNF-alpha receptor. J Virol **77:**7007-16.
15. **Baines, C. P., R. A. Kaiser, N. H. Purcell, N. S. Blair, H. Osinska, M. A. Hambleton, E. W. Brunskill, M. R. Sayen, R. A. Gottlieb, G. W. Dorn, J. Robbins, and J. D. Molkentin.** 2005. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature **434:**658-62.
16. **Baliga, B. C., S. H. Read, and S. Kumar.** 2004. The biochemical mechanism of caspase-2 activation. Cell Death Differ **11:**1234-41.
17. **Baroudy, B. M., S. Venkatesan, and B. Moss.** 1982. Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell **28:**315-24.
18. **Barry, M., and R. C. Bleackley.** 2002. Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol **2:**401-9.
19. **Barry, M., J. A. Heibein, M. J. Pinkoski, S. F. Lee, R. W. Moyer, D. R. Green, and R. C. Bleackley.** 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol **20:**3781-94.

20. **Barry, M., S. T. Wasilenko, T. L. Stewart, and J. M. Taylor.** 2004. Apoptosis regulator genes encoded by poxviruses. Prog Mol Subcell Biol **36:**19-37.

21. **Baxby, D.** 1981. Jenner's Smallpox Vaccine. Heinemann, London.

22. **Bellows, D. S., M. Howell, C. Pearson, S. A. Hazlewood, and J. M. Hardwick.** 2002. Epstein-Barr virus BALF1 is a BCL-2-like antagonist of the herpesvirus antiapoptotic BCL-2 proteins. J Virol **76:**2469-79.

23. **Belzacq, A. S., C. El Hamel, H. L. Vieira, I. Cohen, D. Haouzi, D. Metivier, P. Marchetti, C. Brenner, and G. Kroemer.** 2001. Adenine nucleotide translocator mediates the mitochondrial membrane permeabilization induced by lonidamine, arsenite and CD437. Oncogene **20:**7579-87.

24. **Benedict, C. A., P. S. Norris, T. I. Prigozy, J. L. Bodmer, J. A. Mahr, C. T. Garnett, F. Martinon, J. Tschopp, L. R. Gooding, and C. F. Ware.** 2001. Three adenovirus E3 proteins cooperate to evade apoptosis by tumor necrosis factor-related apoptosis-inducing ligand receptor-1 and -2. J Biol Chem **276:**3270-8.

25. **Bennett, M. J., and D. Eisenberg.** 1994. Refined structure of monomeric diphtheria toxin at 2.3 A resolution. Protein Sci **3:**1464-75.

26. **Bergmann, A., A. Y. Yang, and M. Srivastava.** 2003. Regulators of IAP function: coming to grips with the grim reaper. Curr Opin Cell Biol **15:**717-24.

27. **Bertin, J., R. C. Armstrong, S. Ottilie, D. A. Martin, Y. Wang, S. Banks, G. H. Wang, T. G. Senkevich, E. S. Alnemri, B. Moss, M. J. Lenardo, K. J. Tomaselli, and J. I. Cohen.** 1997. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. Proc Natl Acad Sci U S A **94:**1172-6.

28. **Birnbaum, M. J., R. J. Clem, and L. K. Miller.** 1994. An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. J Virol **68:**2521-8.

29. **Blasco, R., and B. Moss.** 1992. Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. J Virol **66:**4170-9.

30. **Boatright, K. M., M. Renatus, F. L. Scott, S. Sperandio, H. Shin, I. M. Pedersen, J. E. Ricci, W. A. Edris, D. P. Sutherlin, D. R. Green, and G. S. Salvesen.** 2003. A unified model for apical caspase activation. Mol Cell **11:**529-41.

31. **Boehning, D., R. L. Patterson, L. Sedaghat, N. O. Glebova, T. Kurosaki, and S. H. Snyder.** 2003. Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. Nat Cell Biol **5:**1051-61.

32. **Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach.** 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell **85:**803-15.

33. **Boya, P., B. Roques, and G. Kroemer.** 2001. New EMBO members' review: viral and bacterial proteins regulating apoptosis at the mitochondrial level. Embo J **20:**4325-31.

34. **Boya, P., T. Roumier, K. Andreau, R. A. Gonzalez-Polo, N. Zamzami, M. Castedo, and G. Kroemer.** 2003. Mitochondrion-targeted apoptosis regulators of viral origin. Biochem Biophys Res Commun **304:**575-81.

35. **Brdiczka, D.** 1991. Contact sites between mitochondrial envelope membranes. Structure and function in energy- and protein-transfer. Biochim Biophys Acta **1071:**291-312.

36. **Brenner, C., H. Cadiou, H. L. Vieira, N. Zamzami, I. Marzo, Z. Xie, B. Leber, D. Andrews, H. Duclohier, J. C. Reed, and G. Kroemer.** 2000. Bcl-2 and Bax regulate the channel activity of the mitochondrial adenine nucleotide translocator. Oncogene **19:**329-36.

37. **Broyles, S. S.** 2003. Vaccinia virus transcription. J Gen Virol **84:**2293-303.

38. **Brunetti, C. R., H. Amano, Y. Ueda, J. Qin, T. Miyamura, T. Suzuki, X. Li, J. W. Barrett, and G. McFadden.** 2003. Complete genomic sequence and comparative analysis of the tumorigenic poxvirus Yaba monkey tumor virus. J Virol **77:**13335-47.

39. **Brunetti, C. R., M. Paulose-Murphy, R. Singh, J. Qin, J. W. Barrett, A. Tardivel, P. Schneider, K. Essani, and G. McFadden.** 2003. A secreted high-affinity inhibitor of human TNF from Tanapox virus. Proc Natl Acad Sci U S A **100:**4831-6.

40. **Bump, N. J., M. Hackett, M. Hugunin, S. Seshagiri, K. Brady, P. Chen, C. Ferenz, S. Franklin, T. Ghayur, P. Li, and et al.** 1995. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science **269:**1885-8.

41. **Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A **72:**3666-70.

42. **Cartron, P. F., H. Arokium, L. Oliver, K. Meflah, S. Manon, and F. M. Vallette.** 2005. Distinct domains control the addressing and the insertion of Bax into mitochondria. J Biol Chem **280:**10587-98.

43. **Cartron, P. F., T. Gallenne, G. Bougras, F. Gautier, F. Manero, P. Vusio, K. Meflah, F. M. Vallette, and P. Juin.** 2004. The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. Mol Cell **16:**807-18.

44. **Chai, J., E. Shiozaki, S. M. Srinivasula, Q. Wu, P. Datta, E. S. Alnemri, and Y. Shi.** 2001. Structural basis of caspase-7 inhibition by XIAP. Cell **104:**769-80.

45. **Chen, G., and D. V. Goeddel.** 2002. TNF-R1 signaling: a beautiful pathway. Science **296:**1634-5.

46. **Chen, L., S. N. Willis, A. Wei, B. J. Smith, J. I. Fletcher, M. G. Hinds, P. M. Colman, C. L. Day, J. M. Adams, and D. C. Huang.** 2005. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell **17:**393-403.

47. **Cheng, E. H., J. Nicholas, D. S. Bellows, G. S. Hayward, H. G. Guo, M. S. Reitz, and J. M. Hardwick.** 1997. A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak. Proc Natl Acad Sci U S A **94:**690-4.

48. **Cheng, E. H., T. V. Sheiko, J. K. Fisher, W. J. Craigen, and S. J. Korsmeyer.** 2003. VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science **301:**513-7.

49. **Cheng, E. H., M. C. Wei, S. Weiler, R. A. Flavell, T. W. Mak, T. Lindsten, and S. J. Korsmeyer.** 2001. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell **8:**705-11.

50. **Chien, A. J., R. B. Presland, and M. K. Kuechle.** 2002. Processing of native caspase-14 occurs at an atypical cleavage site in normal epidermal differentiation. Biochem Biophys Res Commun **296:**911-7.

51. **Chinnaiyan, A. M., C. G. Tepper, M. F. Seldin, K. O'Rourke, F. C. Kischkel, S. Hellbardt, P. H. Krammer, M. E. Peter, and V. M. Dixit.** 1996. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J Biol Chem **271:**4961-5.

52. **Chiou, S. K., C. C. Tseng, L. Rao, and E. White.** 1994. Functional complementation of the adenovirus E1B 19-kilodalton protein with Bcl-2 in the inhibition of apoptosis in infected cells. J Virol **68:**6553-66.

53. **Chou, C. C., C. Y. Lam, and B. Y. Yung.** 1995. Intracellular ATP is required for actinomycin D-induced apoptotic cell death in HeLa cells. Cancer Lett **96:**181-7.

54. **Cohen, G. M.** 1997. Caspases: the executioners of apoptosis. Biochem J **326 (Pt 1):**1-16.

55. **Cory, S., and J. M. Adams.** 2002. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer **2:**647-56.

56. **Cory, S., D. C. Huang, and J. M. Adams.** 2003. The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene **22:**8590-607.

57. **Crompton, M.** 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem J **341:**233-49.

58. **Crook, N. E., R. J. Clem, and L. K. Miller.** 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol **67:**2168-74.

59. **Cuconati, A., K. Degenhardt, R. Sundararajan, A. Anschel, and E. White.** 2002. Bak and Bax function to limit adenovirus replication through apoptosis induction. J Virol **76:**4547-58.

60. **Cuconati, A., C. Mukherjee, D. Perez, and E. White.** 2003. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. Genes Dev **17:**2922-32.

61. **Cuconati, A., and E. White.** 2002. Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. Genes Dev **16:**2465-78.

62. **Cuervo, A. M.** 2004. Autophagy: in sickness and in health. Trends Cell Biol **14:**70-7.

63. **Cyrklaff, M., C. Risco, J. J. Fernandez, M. V. Jimenez, M. Esteban, W. Baumeister, and J. L. Carrascosa.** 2005. Cryo-electron tomography of vaccinia virus. Proc Natl Acad Sci U S A **102:**2772-7.

64. **Daido, S., T. Kanzawa, A. Yamamoto, H. Takeuchi, Y. Kondo, and S. Kondo.** 2004. Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. Cancer Res **64:**4286-93.

65. **Dales, S., and E. H. Mosbach.** 1968. Vaccinia as a model for membrane biogenesis. Virology **35:**564-83.

66. **Darmon, A. J., T. J. Ley, D. W. Nicholson, and R. C. Bleackley.** 1996. Cleavage of CPP32 by granzyme B represents a critical role for granzyme B in the induction of target cell DNA fragmentation. J Biol Chem **271:**21709-12.

67. **Darmon, A. J., D. W. Nicholson, and R. C. Bleackley.** 1995. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. Nature **377:**446-8.

68. **Degenhardt, K., R. Sundararajan, T. Lindsten, C. Thompson, and E. White.** 2002. Bax and Bak independently promote cytochrome C release from mitochondria. J Biol Chem **277:**14127-34.

69. **Delhon, G., E. R. Tulman, C. L. Afonso, Z. Lu, A. de la Concha-Bermejillo, H. D. Lehmkuhl, M. E. Piccone, G. F. Kutish, and D. L. Rock.** 2004. Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. J Virol **78:**168-77.

70. **Desagher, S., and J. C. Martinou.** 2000. Mitochondria as the central control point of apoptosis. Trends Cell Biol **10:**369-77.

71. **Deveraux, Q. L., and J. C. Reed.** 1999. IAP family proteins--suppressors of apoptosis. Genes Dev **13:**239-52.

72. **Deveraux, Q. L., R. Takahashi, G. S. Salvesen, and J. C. Reed.** 1997. X-linked IAP is a direct inhibitor of cell-death proteases. Nature **388:**300-4.

73. **Dobbelstein, M., and T. Shenk.** 1996. Protection against apoptosis by the vaccinia virus SPI-2 (B13R) gene product. J Virol **70:**6479-85.

74. **Donepudi, M., A. Mac Sweeney, C. Briand, and M. G. Grutter.** 2003. Insights into the regulatory mechanism for caspase-8 activation. Mol Cell **11:**543-9.

75. **Du, C., M. Fang, Y. Li, L. Li, and X. Wang.** 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell **102:**33-42.

76. **Dubochet, J., M. Adrian, K. Richter, J. Garces, and R. Wittek.** 1994. Structure of intracellular mature vaccinia virus observed by cryoelectron microscopy. J Virol **68:**1935-41.

77. **Duvall, E., A. H. Wyllie, and R. G. Morris.** 1985. Macrophage recognition of cells undergoing programmed cell death (apoptosis). Immunology **56:**351-8.

78. **Eckhart, L., J. Ban, H. Fischer, and E. Tschachler.** 2000. Caspase-14: analysis of gene structure and mRNA expression during keratinocyte differentiation. Biochem Biophys Res Commun **277:**655-9.

79. **Edinger, A. L., and C. B. Thompson.** 2004. Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol **16:**663-9.

80. **Eguchi, Y., S. Shimizu, and Y. Tsujimoto.** 1997. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res **57:**1835-40.

81. **Elsing, A., and H. G. Burgert.** 1998. The adenovirus E3/10.4K-14.5K proteins down-modulate the apoptosis receptor Fas/Apo-1 by inducing its internalization. Proc Natl Acad Sci U S A **95:**10072-7.

82. **Eskes, R., B. Antonsson, A. Osen-Sand, S. Montessuit, C. Richter, R. Sadoul, G. Mazzei, A. Nichols, and J. C. Martinou.** 1998. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. J Cell Biol **143:**217-24.

83. **Eskes, R., S. Desagher, B. Antonsson, and J. C. Martinou.** 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol **20:**929-35.

84. **Everett, H., M. Barry, S. F. Lee, X. Sun, K. Graham, J. Stone, R. C. Bleackley, and G. McFadden.** 2000. M11L: a novel mitochondria-localized protein of myxoma virus that blocks apoptosis of infected leukocytes. J Exp Med **191:**1487-98.

85. **Everett, H., M. Barry, X. Sun, S. F. Lee, C. Frantz, L. G. Berthiaume, G. McFadden, and R. C. Bleackley.** 2002. The Myxoma Poxvirus Protein, M11L, Prevents Apoptosis by Direct Interaction with the Mitochondrial Permeability Transition Pore. J Exp Med **196:**1127-1140.

86. **Everett, H., and G. McFadden.** 1999. Apoptosis: an innate immune response to virus infection. Trends Microbiol **7:**160-5.

87. **Feng, P., J. Park, B. S. Lee, S. H. Lee, R. J. Bram, and J. U. Jung.** 2002. Kaposi's sarcoma-associated herpesvirus mitochondrial K7 protein targets a cellular calcium-modulating cyclophilin ligand to modulate intracellular calcium concentration and inhibit apoptosis. J Virol **76:**11491-504.

88. **Feng, P., C. W. Scott, N. H. Cho, H. Nakamura, Y. H. Chung, M. J. Monteiro, and J. U. Jung.** 2004. Kaposi's sarcoma-associated herpesvirus K7 protein targets a ubiquitin-like/ubiquitin-associated domain-containing protein to promote protein degradation. Mol Cell Biol **24:**3938-48.

89. **Fenner, F.** 1981. Mousepox (infectious ectromelia): past, present, and future. Lab Anim Sci **31:**553-9.

90. **Fenner, F.** 1996. Poxviruses, p. 2673-2702. *In* D. M. K. B.N. Fields, P.M Howley et al. (ed.), Fields Virology. Lippincott-raven, Philadelphia.

91. **Fenner, F., D. A. Henderson, I. Arita, A. Jezek, and I. D. Ladnyi.** 1988. Smallpox and its Eradication. World Health Organization publication, Geneva.

92. **Fernandes-Alnemri, T., R. C. Armstrong, J. Krebs, S. M. Srinivasula, L. Wang, F. Bullrich, L. C. Fritz, J. A. Trapani, K. J. Tomaselli, G. Litwack, and E. S. Alnemri.** 1996. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proc Natl Acad Sci U S A **93:**7464-9.

93. **Fessler, S. P., Y. R. Chin, and M. S. Horwitz.** 2004. Inhibition of tumor necrosis factor (TNF) signal transduction by the adenovirus group C RID complex involves downregulation of surface levels of TNF receptor 1. J Virol **78:**13113-21.

94. **Filippova, M., L. Parkhurst, and P. J. Duerksen-Hughes.** 2004. The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. J Biol Chem **279:**25729-44.

95. **Filippova, M., H. Song, J. L. Connolly, T. S. Dermody, and P. J. Duerksen-Hughes.** 2002. The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis. J Biol Chem **277:**21730-9.

96. **Fischer, U., R. U. Janicke, and K. Schulze-Osthoff.** 2003. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ **10:**76-100.

97. **Flint, S., L. Enquist, V. Racaniello, and A. Skalka.** 2003. Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses, 2nd Ed. ed. (ASM) Press, Washington DC.

98. **Frey, T. G., and C. A. Mannella.** 2000. The internal structure of mitochondria. Trends Biochem Sci **25:**319-24.

99. **Frey, T. G., C. W. Renken, and G. A. Perkins.** 2002. Insight into mitochondrial structure and function from electron tomography. Biochim Biophys Acta **1555:**196-203.

100. **Fuentes-Prior, P., and G. S. Salvesen.** 2004. The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J **384:**201-32.

101. **Garvey, T. L., J. Bertin, R. M. Siegel, G. H. Wang, M. J. Lenardo, and J. I. Cohen.** 2002. Binding of FADD and caspase-8 to molluscum contagiosum virus MC159 v- FLIP is not sufficient for its antiapoptotic function. J Virol **76:**697-706.

102. **Germain, M., J. P. Mathai, H. M. McBride, and G. C. Shore.** 2005. Endoplasmic reticulum BIK initiates DRP1-regulated remodelling of mitochondrial cristae during apoptosis. Embo J.

103. **Geshelin, P., and K. I. Berns.** 1974. Characterization and localization of the naturally occurring cross-links in vaccinia virus DNA. J Mol Biol **88:**785-96.

104. **Gettins, P. G.** 2002. Serpin structure, mechanism, and function. Chem Rev **102:**4751-804.

105. **Goebel, S. J., G. P. Johnson, M. E. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete DNA sequence of vaccinia virus. Virology **179:**247-66, 517-63.

106. **Goldmacher, V. S., L. M. Bartle, A. Skaletskaya, C. A. Dionne, N. L. Kedersha, C. A. Vater, J. W. Han, R. J. Lutz, S. Watanabe, E. D. Cahir McFarland, E. D. Kieff, E. S. Mocarski, and T. Chittenden.** 1999. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc Natl Acad Sci U S A **96:**12536-41.

107. **Goldstein, J. C., N. J. Waterhouse, P. Juin, G. I. Evan, and D. R. Green.** 2000. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. Nat Cell Biol **2:**156-62.

108. **Goodpasture, E. W., A. M. Woodruff, and G. J. Buddingh.** 1932. Vaccinial infection of chorioallantoic membrane of chick embryo. Am. J. Pathol. **8:**271-282.

109. **Goping, I. S., M. Barry, P. Liston, T. Sawchuk, G. Constantinescu, K. M. Michalak, I. Shostak, D. L. Roberts, A. M. Hunter, R. Korneluk, and R. C. Bleackley.** 2003. Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. Immunity **18:**355-65.

110. **Gottlieb, E., S. M. Armour, M. H. Harris, and C. B. Thompson.** 2003. Mitochondrial membrane potential regulates matrix configuration and cytochrome c release during apoptosis. Cell Death Differ **10:**709-17.

111. **Gozuacik, D., and A. Kimchi.** 2004. Autophagy as a cell death and tumor suppressor mechanism. Oncogene **23:**2891-906.

112. **Green, D. R., and G. I. Evan.** 2002. A matter of life and death. Cancer Cell **1:**19-30.

113. **Green, D. R., and J. C. Reed.** 1998. Mitochondria and apoptosis. Science **281:**1309-12.

114. **Griffiths, G., N. Roos, S. Schleich, and J. K. Locker.** 2001. Structure and assembly of intracellular mature vaccinia virus: thin-section analyses. J Virol **75:**11056-70.

115. **Griffiths, G. J., B. M. Corfe, P. Savory, S. Leech, M. D. Esposti, J. A. Hickman, and C. Dive.** 2001. Cellular damage signals promote sequential changes at the N-terminus and BH-1 domain of the pro-apoptotic protein Bak. Oncogene **20:**7668-76.

116. **Griffiths, G. J., L. Dubrez, C. P. Morgan, N. A. Jones, J. Whitehouse, B. M. Corfe, C. Dive, and J. A. Hickman.** 1999. Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. J Cell Biol **144:**903-14.

117. **Gross, A., J. M. McDonnell, and S. J. Korsmeyer.** 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev **13:**1899-911.

118. **Gubser, C., S. Hue, P. Kellam, and G. L. Smith.** 2004. Poxvirus genomes: a phylogenetic analysis. J Gen Virol **85:**105-17.

119. **Guo, B., D. Zhai, E. Cabezas, K. Welsh, S. Nouraini, A. C. Satterthwait, and J. C. Reed.** 2003. Humanin peptide suppresses apoptosis by interfering with Bax activation. Nature **423:**456-61.

120. **Han, J., L. A. Goldstein, B. R. Gastman, C. J. Froelich, X. M. Yin, and H. Rabinowich.** 2004. Degradation of Mcl-1 by granzyme B: implications for Bim-mediated mitochondrial apoptotic events. J Biol Chem **279:**22020-9.

121. **Han, J., L. A. Goldstein, B. R. Gastman, A. Rabinovitz, and H. Rabinowich.** 2005. Disruption of MCL-1/BIM complex in granzyme B-mediated mitochondrial apoptosis. J Biol Chem.

122. **Hardwick, J. M., and D. S. Bellows.** 2003. Viral versus cellular BCL-2 proteins. Cell Death Differ **10 Suppl 1:**S68-76.

123. **Harris, J. L., E. P. Peterson, D. Hudig, N. A. Thornberry, and C. S. Craik.** 1998. Definition and redesign of the extended substrate specificity of granzyme B. J Biol Chem **273:**27364-73.

124. **Hawkins, C. J., S. J. Yoo, E. P. Peterson, S. L. Wang, S. Y. Vernooy, and B. A. Hay.** 2000. The Drosophila caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. J Biol Chem **275:**27084-93.

125. **Hayajneh, W. A., A. M. Colberg-Poley, A. Skaletskaya, L. M. Bartle, M. M. Lesperance, D. G. Contopoulos-Ioannidis, N. L. Kedersha, and V. S. Goldmacher.** 2001. The sequence and antiapoptotic functional domains of the human cytomegalovirus UL37 exon 1 immediate early protein are conserved in multiple primary strains. Virology **279:**233-40.

126. **Hegde, R., S. M. Srinivasula, Z. Zhang, R. Wassell, R. Mukattash, L. Cilenti, G. DuBois, Y. Lazebnik, A. S. Zervos, T. Fernandes-Alnemri, and E. S. Alnemri.** 2002. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J Biol Chem **277:**432-8.

127. **Heibein, J. A., M. Barry, B. Motyka, and R. C. Bleackley.** 1999. Granzyme B-induced loss of mitochondrial inner membrane potential (Delta Psi m) and cytochrome c release are caspase independent. J Immunol **163:**4683-93.

128. **Heibein, J. A., I. S. Goping, M. Barry, M. J. Pinkoski, G. C. Shore, D. R. Green, and R. C. Bleackley.** 2000. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. J Exp Med **192:**1391-402.

129. **Higuchi, M., T. Honda, R. J. Proske, and E. T. Yeh.** 1998. Regulation of reactive oxygen species-induced apoptosis and necrosis by caspase 3-like proteases. Oncogene **17:**2753-60.

130. **Hilgendorf, A., J. Lindberg, Z. Ruzsics, S. Honing, A. Elsing, M. Lofqvist, H. Engelmann, and H. G. Burgert.** 2003. Two distinct transport motifs in the adenovirus E3/10.4-14.5 proteins

act in concert to down-modulate apoptosis receptors and the epidermal growth factor receptor. J Biol Chem **278**:51872-84.

131. **Hiller, G., and K. Weber.** 1985. Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. J Virol **55**:651-9.

132. **Hockenbery, D., G. Nunez, C. Milliman, R. D. Schreiber, and S. J. Korsmeyer.** 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature **348**:334-6.

133. **Hollinshead, M., A. Vanderplasschen, G. L. Smith, and D. J. Vaux.** 1999. Vaccinia virus intracellular mature virions contain only one lipid membrane. J Virol **73**:1503-17.

134. **Holowczak, J. A.** 1972. Uncoating of poxviruses. I. Detection and characterization of subviral particles in the uncoating process. Virology **50**:216-32.

135. **Hsu, Y. T., and R. J. Youle.** 1997. Nonionic detergents induce dimerization among members of the Bcl-2 family. J Biol Chem **272**:13829-34.

136. **Hu, F. Q., C. A. Smith, and D. J. Pickup.** 1994. Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. Virology **204**:343-56.

137. **Hu, S., C. Vincenz, M. Buller, and V. M. Dixit.** 1997. A novel family of viral death effector domain-containing molecules that inhibit both CD-95- and tumor necrosis factor receptor-1-induced apoptosis. J Biol Chem **272**:9621-4.

138. **Huang, D. C., and A. Strasser.** 2000. BH3-Only proteins-essential initiators of apoptotic cell death. Cell **103**:839-42.

139. **Huang, Q., A. M. Petros, H. W. Virgin, S. W. Fesik, and E. T. Olejniczak.** 2002. Solution structure of a Bcl-2 homolog from Kaposi sarcoma virus. Proc Natl Acad Sci U S A **99**:3428-33.

140. **Huang, Q., A. M. Petros, H. W. Virgin, S. W. Fesik, and E. T. Olejniczak.** 2003. Solution structure of the BHRF1 protein from Epstein-Barr virus, a homolog of human Bcl-2. J Mol Biol **332**:1123-30.

141. **Huang, Y., Y. C. Park, R. L. Rich, D. Segal, D. G. Myszka, and H. Wu.** 2001. Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. Cell **104**:781-90.

142. **Ichas, F., and J. P. Mazat.** 1998. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim Biophys Acta **1366**:33-50.

143. **Jaattela, M., and J. Tschopp.** 2003. Caspase-independent cell death in T lymphocytes. Nat Immunol **4**:416-23.

144. **Jabbour, A. M., P. G. Ekert, E. J. Coulson, M. J. Knight, D. M. Ashley, and C. J. Hawkins.** 2002. The p35 relative, p49, inhibits mammalian and Drosophila caspases including DRONC and protects against apoptosis. Cell Death Differ **9**:1311-20.

145. **Johnston, J. B., and G. McFadden.** 2003. Poxvirus immunomodulatory strategies: current perspectives. J Virol **77**:6093-100.

146. **Johnstone, R. W., A. A. Ruefli, and S. W. Lowe.** 2002. Apoptosis: a link between cancer genetics and chemotherapy. Cell **108**:153-64.

147. **Joklik, W. K.** 1964. The Intracellular Uncoating of Poxvirus DNA. Ii. The Molecular Basis of the Uncoating Process. J Mol Biol **78**:277-88.

148. **Kabsch, K., and A. Alonso.** 2002. The human papillomavirus type 16 E5 protein impairs TRAIL- and FasL-mediated apoptosis in HaCaT cells by different mechanisms. J Virol **76**:12162-72.

149. **Kanduc, D., A. Mittelman, R. Serpico, E. Sinigaglia, A. A. Sinha, C. Natale, R. Santacroce, M. G. Di Corcia, A. Lucchese, L. Dini, P. Pani, S. Santacroce, S. Simone, R. Bucci, and E. Farber.** 2002. Cell death: apoptosis versus necrosis (review). Int J Oncol **21**:165-70.

150. **Kanzawa, T., I. M. Germano, T. Komata, H. Ito, Y. Kondo, and S. Kondo.** 2004. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ **11**:448-57.

151. **Karbowski, M., Y. J. Lee, B. Gaume, S. Y. Jeong, S. Frank, A. Nechushtan, A. Santel, M. Fuller, C. L. Smith, and R. J. Youle.** 2002. Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J Cell Biol **159**:931-8.

152. **Kates, J., and J. Beeson.** 1970. Ribonucleic acid synthesis in vaccinia virus. I. The mechanism of synthesis and release of RNA in vaccinia cores. J Mol Biol **50**:1-18.

153. **Kerr, J. F., A. H. Wyllie, and A. R. Currie.** 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer **26**:239-57.

154. **Kettle, S., A. Alcami, A. Khanna, R. Ehret, C. Jassoy, and G. L. Smith.** 1997. Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1beta-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1beta-induced fever. J Gen Virol **78 (Pt 3)**:677-85.

155. **Kischkel, F. C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P. H. Krammer, and M. E. Peter.** 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. Embo J **14**:5579-88.

156. **Kleijnen, M. F., A. H. Shih, P. Zhou, S. Kumar, R. E. Soccio, N. L. Kedersha, G. Gill, and P. M. Howley.** 2000. The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome. Mol Cell **6**:409-19.

157. **Kluck, R. M., M. D. Esposti, G. Perkins, C. Renken, T. Kuwana, E. Bossy-Wetzel, M. Goldberg, T. Allen, M. J. Barber, D. R. Green, and D. D. Newmeyer.** 1999. The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. J Cell Biol **147**:809-22.

158. **Komiyama, T., C. A. Ray, D. J. Pickup, A. D. Howard, N. A. Thornberry, E. P. Peterson, and G. Salvesen.** 1994. Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. J Biol Chem **269**:19331-7.

159. **Kozopas, K. M., T. Yang, H. L. Buchan, P. Zhou, and R. W. Craig.** 1993. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci U S A **90**:3516-20.

160. **Kroemer, G., B. Dallaporta, and M. Resche-Rigon.** 1998. The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol **60**:619-42.

161. **Kroemer, G., and J. C. Reed.** 2000. Mitochondrial control of cell death. Nat Med **6**:513-9.

162. **Kroemer, G., N. Zamzami, and S. A. Susin.** 1997. Mitochondrial control of apoptosis. Immunol Today **18**:44-51.

163. **Kumar, S., and J. Doumanis.** 2000. The fly caspases. Cell Death Differ **7**:1039-44.

164. **Kuwana, T., L. Bouchier-Hayes, J. E. Chipuk, C. Bonzon, B. A. Sullivan, D. R. Green, and D. D. Newmeyer.** 2005. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol Cell **17**:525-35.

165. **Kuwana, T., M. R. Mackey, G. Perkins, M. H. Ellisman, M. Latterich, R. Schneiter, D. R. Green, and D. D. Newmeyer.** 2002. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell **111**:331-42.

166. **Lanave, C., M. Santamaria, and C. Saccone.** 2004. Comparative genomics: the evolutionary history of the Bcl-2 family. Gene **333**:71-9.

167. **Lee, H. J., K. Essani, and G. L. Smith.** 2001. The genome sequence of Yaba-like disease virus, a yatapoxvirus. Virology **281**:170-92.

168. **Leist, M., B. Single, A. F. Castoldi, S. Kuhnle, and P. Nicotera.** 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med **185**:1481-6.

169. **Letai, A., M. C. Bassik, L. D. Walensky, M. D. Sorcinelli, S. Weiler, and S. J. Korsmeyer.** 2002. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell **2**:183-92.

170. **Leu, J. I., P. Dumont, M. Hafey, M. E. Murphy, and D. L. George.** 2004. Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol **6**:443-50.

171. **Levine, B.** 2005. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. Cell **120**:159-62.

172. **Lewis-Jones, S.** 2004. Zoonotic poxvirus infections in humans. Curr Opin Infect Dis **17**:81-9.

173. **Li, H., H. Zhu, C. J. Xu, and J. Yuan.** 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell **94**:491-501.

174. **Li, L. Y., X. Luo, and X. Wang.** 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature **412**:95-9.

175. **Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang.** 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell **91**:479-89.

176. **Li, Q., P. Liston, and R. W. Moyer.** 2005. Functional analysis of the inhibitor of apoptosis (iap) gene carried by the entomopoxvirus of Amsacta moorei. J Virol **79**:2335-45.

177. **Li, Q., P. Liston, N. Schokman, J. M. Ho, and R. W. Moyer.** 2005. Amsacta moorei Entomopoxvirus inhibitor of apoptosis suppresses cell death by binding Grim and Hid. J Virol **79:**3684-91.

178. **Lichtenstein, D. L., K. Doronin, K. Toth, M. Kuppuswamy, W. S. Wold, and A. E. Tollefson.** 2004. Adenovirus E3-6.7K protein is required in conjunction with the E3-RID protein complex for the internalization and degradation of TRAIL receptor 2. J Virol **78:**12297-307.

179. **Lichtenstein, D. L., P. Krajcsi, D. J. Esteban, A. E. Tollefson, and W. S. Wold.** 2002. Adenovirus RIDbeta subunit contains a tyrosine residue that is critical for RID-mediated receptor internalization and inhibition of Fas- and TRAIL-induced apoptosis. J Virol **76:**11329-42.

180. **Lindsten, T., A. J. Ross, A. King, W. X. Zong, J. C. Rathmell, H. A. Shiels, E. Ulrich, K. G. Waymire, P. Mahar, K. Frauwirth, Y. Chen, M. Wei, V. M. Eng, D. M. Adelman, M. C. Simon, A. Ma, J. A. Golden, G. Evan, S. J. Korsmeyer, G. R. MacGregor, and C. B. Thompson.** 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell **6:**1389-99.

181. **Lippens, S., M. Kockx, M. Knaapen, L. Mortier, R. Polakowska, A. Verheyen, M. Garmyn, A. Zwijsen, P. Formstecher, D. Huylebroeck, P. Vandenabeele, and W. Declercq.** 2000. Epidermal differentiation does not involve the pro-apoptotic executioner caspases, but is associated with caspase-14 induction and processing. Cell Death Differ **7:**1218-24.

182. **Liu, X., S. Dai, Y. Zhu, P. Marrack, and J. W. Kappler.** 2003. The structure of a Bcl-xL/Bim fragment complex: implications for Bim function. Immunity **19:**341-52.

183. **Liu, X., C. N. Kim, J. Yang, R. Jemmerson, and X. Wang.** 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86:**147-57.

184. **Locker, J. K., A. Kuehn, S. Schleich, G. Rutter, H. Hohenberg, R. Wepf, and G. Griffiths.** 2000. Entry of the two infectious forms of vaccinia virus at the plasma membane is signaling-dependent for the IMV but not the EEV. Mol Biol Cell **11:**2497-511.

185. **Lockshin, R. A., and Z. Zakeri.** 2004. Apoptosis, autophagy, and more. Int J Biochem Cell Biol **36:**2405-19.

186. **Lodish, H., D. Baltimore, A. Berk, S. Zipursky, P. Matsudaira, and J. Darnell.** 1995. Organell Biogenesis: The mitochondrion, chloroplast, peroxisome, and nucleus., p. 809-849. *In* J. Darnell (ed.), Molecular Cell Biology, 3rd edition ed. Scientific American Books, Inc., New York.

187. **Loparev, V. N., J. M. Parsons, J. C. Knight, J. F. Panus, C. A. Ray, R. M. Buller, D. J. Pickup, and J. J. Esposito.** 1998. A third distinct tumor necrosis factor receptor of orthopoxviruses. Proc Natl Acad Sci U S A **95:**3786-91.

188. **Lum, J. J., D. E. Bauer, M. Kong, M. H. Harris, C. Li, T. Lindsten, and C. B. Thompson.** 2005. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell **120:**237-48.

189. **Luo, X., I. Budihardjo, H. Zou, C. Slaughter, and X. Wang.** 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell **94:**481-90.

190. **Macen, J. L., R. S. Garner, P. Y. Musy, M. A. Brooks, P. C. Turner, R. W. Moyer, G. McFadden, and R. C. Bleackley.** 1996. Differential inhibition of the Fas- and granule-mediated cytolysis pathways by the orthopoxvirus cytokine response modifier A/SPI-2 and SPI-1 protein. Proc Natl Acad Sci U S A **93:**9108-13.

191. **Mackett, M., G. L. Smith, and B. Moss.** 1982. Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc Natl Acad Sci U S A **79:**7415-9.

192. **Madeo, F., E. Herker, C. Maldener, S. Wissing, S. Lachelt, M. Herlan, M. Fehr, K. Lauber, S. J. Sigrist, S. Wesselborg, and K. U. Frohlich.** 2002. A caspase-related protease regulates apoptosis in yeast. Mol Cell **9:**911-7.

193. **Majno, G., and I. Joris.** 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol **146:**3-15.

194. **Majumder, M., A. K. Ghosh, R. Steele, X. Y. Zhou, N. J. Phillips, R. Ray, and R. B. Ray.** 2002. Hepatitis C virus NS5A protein impairs TNF-mediated hepatic apoptosis, but not by an anti-FAS antibody, in transgenic mice. Virology **294:**94-105.

195. **Mannella, C. A., M. Marko, and K. Buttle.** 1997. Reconsidering mitochondrial structure: new views of an old organelle. Trends Biochem Sci **22:**37-8.

196. **Mannella, C. A., M. Marko, P. Penczek, D. Barnard, and J. Frank.** 1994. The internal compartmentation of rat-liver mitochondria: tomographic study using the high-voltage transmission electron microscope. Microsc Res Tech **27:**278-83.

197. **Mannella, C. A., D. R. Pfeiffer, P. C. Bradshaw, Moraru, II, B. Slepchenko, L. M. Loew, C. E. Hsieh, K. Buttle, and M. Marko.** 2001. Topology of the mitochondrial inner membrane: dynamics and bioenergetic implications. IUBMB Life **52:**93-100.

198. **Marchetti, P., T. Hirsch, N. Zamzami, M. Castedo, D. Decaudin, S. A. Susin, B. Masse, and G. Kroemer.** 1996. Mitochondrial permeability transition triggers lymphocyte apoptosis. J Immunol **157:**4830-6.

199. **Marino, G., and C. Lopez-Otin.** 2004. Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. Cell Mol Life Sci **61:**1439-54.

200. **Martin, S. J., G. P. Amarante-Mendes, L. Shi, T. H. Chuang, C. A. Casiano, G. A. O'Brien, P. Fitzgerald, E. M. Tan, G. M. Bokoch, A. H. Greenberg, and D. R. Green.** 1996. The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. Embo J **15:**2407-16.

201. **Martinou, J. C., S. Desagher, and B. Antonsson.** 2000. Cytochrome c release from mitochondria: all or nothing. Nat Cell Biol **2:**E41-3.

202. **Martinou, J. C., and D. R. Green.** 2001. Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol **2:**63-7.

203. **Marzo, I., C. Brenner, N. Zamzami, J. M. Jurgensmeier, S. A. Susin, H. L. Vieira, M. C. Prevost, Z. Xie, S. Matsuyama, J. C. Reed, and G. Kroemer.** 1998. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science **281:**2027-31.

204. **Massung, R. F., J. J. Esposito, L. I. Liu, J. Qi, T. R. Utterback, J. C. Knight, L. Aubin, T. E. Yuran, J. M. Parsons, V. N. Loparev, and et al.** 1993. Potential virulence determinants in terminal regions of variola smallpox virus genome. Nature **366:**748-51.

205. **Massung, R. F., L. I. Liu, J. Qi, J. C. Knight, T. E. Yuran, A. R. Kerlavage, J. M. Parsons, J. C. Venter, and J. J. Esposito.** 1994. Analysis of the complete genome of smallpox variola major virus strain Bangladesh-1975. Virology **201:**215-40.

206. **Mathews, C., and K. Van Holde.** 1996. Biological Oxidations, Electron Transport, and Oxidative Phosphorylation, p. 520-553. *In* L. With (ed.), Biochemistry, Second Edition ed. The Benjamin/Cummings Publishing Company, Inc., Menlo Park.

207. **McCormick, A. L., A. Skaletskaya, P. A. Barry, E. S. Mocarski, and V. S. Goldmacher.** 2003. Differential function and expression of the viral inhibitor of caspase 8-induced apoptosis (vICA) and the viral mitochondria-localized inhibitor of apoptosis (vMIA) cell death suppressors conserved in primate and rodent cytomegaloviruses. Virology **316:**221-33.

208. **McCormick, A. L., V. L. Smith, D. Chow, and E. S. Mocarski.** 2003. Disruption of mitochondrial networks by the human cytomegalovirus UL37 gene product viral mitochondrion-localized inhibitor of apoptosis. J Virol **77:**631-41.

209. **McDonnell, J. M., D. Fushman, C. L. Milliman, S. J. Korsmeyer, and D. Cowburn.** 1999. Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. Cell **96:**625-34.

210. **McDonnell, T. J., N. Deane, F. M. Platt, G. Nunez, U. Jaeger, J. P. McKearn, and S. J. Korsmeyer.** 1989. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell **57:**79-88.

211. **Meier, P., J. Silke, S. J. Leevers, and G. I. Evan.** 2000. The Drosophila caspase DRONC is regulated by DIAP1. Embo J **19:**598-611.

212. **Meijer, A. J., and P. Codogno.** 2004. Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol **36:**2445-62.

213. **Messud-Petit, F., J. Gelfi, M. Delverdier, M. F. Amardeilh, R. Py, G. Sutter, and S. Bertagnoli.** 1998. Serp2, an inhibitor of the interleukin-1beta-converting enzyme, is critical in the pathobiology of myxoma virus. J Virol **72:**7830-9.

214. **Metkar, S. S., B. Wang, M. L. Ebbs, J. H. Kim, Y. J. Lee, S. M. Raja, and C. J. Froelich.** 2003. Granzyme B activates procaspase-3 which signals a mitochondrial amplification loop for maximal apoptosis. J Cell Biol **160:**875-85.

215. **Miyasaka, Y., N. Enomoto, M. Kurosaki, N. Sakamoto, N. Kanazawa, T. Kohashi, E. Ueda, S. Maekawa, H. Watanabe, N. Izumi, C. Sato, and M. Watanabe.** 2003. Hepatitis C virus nonstructural protein 5A inhibits tumor necrosis factor-alpha-mediated apoptosis in Huh7 cells. J Infect Dis **188:**1537-44.

216. **Moss, B.** 1996. Poxviridae: The Viruses and Their Replication, p. 2637-2671. *In* D. M. K. B.N. Fields, P.M Howley et al. (ed.), Fields Virology. Lippincott-Raven, Philadelphia.

217. **Moss, B., and E. N. Rosenblum.** 1973. Letter: Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. J Mol Biol **81:**267-9.

218. **Muchmore, S. W., M. Sattler, H. Liang, R. P. Meadows, J. E. Harlan, H. S. Yoon, D. Nettesheim, B. S. Chang, C. B. Thompson, S. L. Wong, S. L. Ng, and S. W. Fesik.** 1996. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature **381:**335-41.

219. **Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Krammer, M. E. Peter, and V. M. Dixit.** 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell **85:**817-27.

220. **Nagington, J., and R. W. Horne.** 1962. Morphological studies of orf and vaccinia viruses. Virology **16:**248-60.

221. **Nakagawa, T., S. Shimizu, T. Watanabe, O. Yamaguchi, K. Otsu, H. Yamagata, H. Inohara, T. Kubo, and Y. Tsujimoto.** 2005. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature **434:**652-8.

222. **Nakai, M., A. Takeda, M. L. Cleary, and T. Endo.** 1993. The bcl-2 protein is inserted into the outer membrane but not into the inner membrane of rat liver mitochondria in vitro. Biochem Biophys Res Commun **196:**233-9.

223. **Nava, V. E., E. H. Cheng, M. Veliuona, S. Zou, R. J. Clem, M. L. Mayer, and J. M. Hardwick.** 1997. Herpesvirus saimiri encodes a functional homolog of the human bcl-2 oncogene. J Virol **71:**4118-22.

224. **Neznanov, N., A. Kondratova, K. M. Chumakov, B. Angres, B. Zhumabayeva, V. I. Agol, and A. V. Gudkov.** 2001. Poliovirus protein 3A inhibits tumor necrosis factor (TNF)-induced apoptosis by eliminating the TNF receptor from the cell surface. J Virol **75:**10409-20.

225. **Nguyen, M., D. G. Millar, V. W. Yong, S. J. Korsmeyer, and G. C. Shore.** 1993. Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. J Biol Chem **268:**25265-8.

226. **Nicholson, D. W., and N. A. Thornberry.** 1997. Caspases: killer proteases. Trends Biochem Sci **22:**299-306.

227. **Nijhawan, D., M. Fang, E. Traer, Q. Zhong, W. Gao, F. Du, and X. Wang.** 2003. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev **17:**1475-86.

228. **Nogal, M. L., G. Gonzalez de Buitrago, C. Rodriguez, B. Cubelos, A. L. Carrascosa, M. L. Salas, and Y. Revilla.** 2001. African swine fever virus IAP homologue inhibits caspase activation and promotes cell survival in mammalian cells. J Virol **75:**2535-43.

229. **Nomura, M., S. Shimizu, T. Sugiyama, M. Narita, T. Ito, H. Matsuda, and Y. Tsujimoto.** 2003. 14-3-3 Interacts directly with and negatively regulates pro-apoptotic Bax. J Biol Chem **278:**2058-65.

230. **Ogier-Denis, E., and P. Codogno.** 2003. Autophagy: a barrier or an adaptive response to cancer. Biochim Biophys Acta **1603:**113-28.

231. **Okada, M., S. Adachi, T. Imai, K. Watanabe, S. Y. Toyokuni, M. Ueno, A. S. Zervos, G. Kroemer, and T. Nakahata.** 2004. A novel mechanism for imatinib mesylate-induced cell death of BCR-ABL-positive human leukemic cells: caspase-independent, necrosis-like programmed cell death mediated by serine protease activity. Blood **103:**2299-307.

232. **Opferman, J. T., and S. J. Korsmeyer.** 2003. Apoptosis in the development and maintenance of the immune system. Nat Immunol **4:**410-5.

233. **Paglin, S., T. Hollister, T. Delohery, N. Hackett, M. McMahill, E. Sphicas, D. Domingo, and J. Yahalom.** 2001. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res **61:**439-44.

234. **Palmer, E.** 2003. Negative selection--clearing out the bad apples from the T-cell repertoire. Nat Rev Immunol **3:**383-91.

235. **Panicali, D., and E. Paoletti.** 1982. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad Sci U S A **79:**4927-31.

236. **Park, K. J., S. H. Choi, S. Y. Lee, S. B. Hwang, and M. M. Lai.** 2002. Nonstructural 5A protein of hepatitis C virus modulates tumor necrosis factor alpha-stimulated nuclear factor kappa B activation. J Biol Chem **277:**13122-8.

237. **Parker, M. W., J. P. Postma, F. Pattus, A. D. Tucker, and D. Tsernoglou.** 1992. Refined structure of the pore-forming domain of colicin A at 2.4 A resolution. J Mol Biol **224:**639-57.

238. **Patterson, S. D., C. S. Spahr, E. Daugas, S. A. Susin, T. Irinopoulou, C. Koehler, and G. Kroemer.** 2000. Mass spectrometric identification of proteins released from mitochondria undergoing permeability transition. Cell Death Differ **7:**137-44.

239. **Paulose, M., B. L. Bennett, A. M. Manning, and K. Essani.** 1998. Selective inhibition of TNF-alpha induced cell adhesion molecule gene expression by tanapox virus. Microb Pathog **25:**33-41.

240. **Payne, L. G.** 1980. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J Gen Virol **50:**89-100.

241. **Payne, L. G., and E. Norrby.** 1978. Adsorption and penetration of enveloped and naked vaccinia virus particles. J Virol **27:**19-27.

242. **Perez, D., and E. White.** 2000. TNF-alpha signals apoptosis through a bid-dependent conformational change in Bax that is inhibited by E1B 19K. Mol Cell **6:**53-63.

243. **Perfettini, J. L., T. Roumier, and G. Kroemer.** 2005. Mitochondrial fusion and fission in the control of apoptosis. Trends Cell Biol **15:**179-83.

244. **Perkus, M. E., S. J. Goebel, S. W. Davis, G. P. Johnson, E. K. Norton, and E. Paoletti.** 1991. Deletion of 55 open reading frames from the termini of vaccinia virus. Virology **180:**406-10.

245. **Peter, M. E., and P. H. Krammer.** 2003. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ **10:**26-35.

246. **Petit, F., S. Bertagnoli, J. Gelfi, F. Fassy, C. Boucraut-Baralon, and A. Milon.** 1996. Characterization of a myxoma virus-encoded serpin-like protein with activity against interleukin-1 beta-converting enzyme. J Virol **70:**5860-6.

247. **Petit, P. X., H. Lecoeur, E. Zorn, C. Dauguet, B. Mignotte, and M. L. Gougeon.** 1995. Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. J Cell Biol **130:**157-67.

248. **Petros, A. M., A. Medek, D. G. Nettesheim, D. H. Kim, H. S. Yoon, K. Swift, E. D. Matayoshi, T. Oltersdorf, and S. W. Fesik.** 2001. Solution structure of the antiapoptotic protein bcl-2. Proc Natl Acad Sci U S A **98:**3012-7.

249. **Petros, A. M., E. T. Olejniczak, and S. W. Fesik.** 2004. Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta **1644:**83-94.

250. **Pickup, D. J., B. S. Ink, W. Hu, C. A. Ray, and W. K. Joklik.** 1986. Hemorrhage in lesions caused by cowpox virus is induced by a viral protein that is related to plasma protein inhibitors of serine proteases. Proc Natl Acad Sci U S A **83:**7698-702.

251. **Poncet, D., P. Boya, D. Metivier, N. Zamzami, and G. Kroemer.** 2003. Cytofluorometric quantitation of apoptosis-driven inner mitochondrial membrane permeabilization. Apoptosis **8:**521-30.

252. **Poncet, D., N. Larochette, A. L. Pauleau, P. Boya, A. A. Jalil, P. F. Cartron, F. Vallette, C. Schnebelen, L. M. Bartle, A. Skaletskaya, D. Boutolleau, J. C. Martinou, V. S. Goldmacher, G. Kroemer, and N. Zamzami.** 2004. An anti-apoptotic viral protein that recruits Bax to mitochondria. J Biol Chem **279:**22605-14.

253. **Preston, R.** 2002. The demon in the freezer. The Random House Publishing Group.

254. **Puthalakath, H., and A. Strasser.** 2002. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ **9:**505-12.

255. **Quan, L. T., A. Caputo, R. C. Bleackley, D. J. Pickup, and G. S. Salvesen.** 1995. Granzyme B is inhibited by the cowpox virus serpin cytokine response modifier A. J Biol Chem **270:**10377-9.

256. **Quan, L. T., M. Tewari, K. O'Rourke, V. Dixit, S. J. Snipas, G. G. Poirier, C. Ray, D. J. Pickup, and G. S. Salvesen.** 1996. Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B. Proc Natl Acad Sci U S A **93:**1972-6.

257. **Ranger, A. M., B. A. Malynn, and S. J. Korsmeyer.** 2001. Mouse models of cell death. Nat Genet **28:**113-8.

258. **Rano, T. A., T. Timkey, E. P. Peterson, J. Rotonda, D. W. Nicholson, J. W. Becker, K. T. Chapman, and N. A. Thornberry.** 1997. A combinatorial approach for determining protease specificities: application to interleukin-1beta converting enzyme (ICE). Chem Biol **4:**149-55.

259. **Rathmell, J. C., and C. B. Thompson.** 1999. The central effectors of cell death in the immune system. Annu Rev Immunol **17:**781-828.

260. **Ray, C. A., R. A. Black, S. R. Kronheim, T. A. Greenstreet, P. R. Sleath, G. S. Salvesen, and D. J. Pickup.** 1992. Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. Cell **69:**597-604.

261. **Reading, P. C., A. Khanna, and G. L. Smith.** 2002. Vaccinia virus CrmE encodes a soluble and cell surface tumor necrosis factor receptor that contributes to virus virulence. Virology **292:**285-98.

262. **Reboredo, M., R. F. Greaves, and G. Hahn.** 2004. Human cytomegalovirus proteins encoded by UL37 exon 1 protect infected fibroblasts against virus-induced apoptosis and are required for efficient virus replication. J Gen Virol **85:**3555-67.

263. **Richter, C., M. Schweizer, A. Cossarizza, and C. Franceschi.** 1996. Control of apoptosis by the cellular ATP level. FEBS Lett **378:**107-10.

264. **Riedl, S. J., M. Renatus, R. Schwarzenbacher, Q. Zhou, C. Sun, S. W. Fesik, R. C. Liddington, and G. S. Salvesen.** 2001. Structural basis for the inhibition of caspase-3 by XIAP. Cell **104:**791-800.

265. **Roulston, A., R. C. Marcellus, and P. E. Branton.** 1999. Viruses and apoptosis. Annu Rev Microbiol **53:**577-628.

266. **Ruffolo, S. C., and G. C. Shore.** 2003. BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization. J Biol Chem **278:**25039-45.

267. **Ruska, H., and G. A. Kausche.** 1943. Über Form, Grössenverteilung und Struktur einiger Virus-Elementarkörper. Zbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **150:**311-318.

268. **Russell, J. H., and T. J. Ley.** 2002. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol **20:**323-70.

269. **Salvesen, G. S., and V. M. Dixit.** 1999. Caspase activation: the induced-proximity model. Proc Natl Acad Sci U S A **96:**10964-7.

270. **Salvesen, G. S., and C. S. Duckett.** 2002. IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol **3:**401-10.

271. **Saraiva, M., and A. Alcami.** 2001. CrmE, a novel soluble tumor necrosis factor receptor encoded by poxviruses. J Virol **75:**226-33.

272. **Sattler, M., H. Liang, D. Nettesheim, R. P. Meadows, J. E. Harlan, M. Eberstadt, H. S. Yoon, S. B. Shuker, B. S. Chang, A. J. Minn, C. B. Thompson, and S. W. Fesik.** 1997. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science **275:**983-6.

273. **Sawada, M., W. Sun, P. Hayes, K. Leskov, D. A. Boothman, and S. Matsuyama.** 2003. Ku70 suppresses the apoptotic translocation of Bax to mitochondria. Nat Cell Biol **5:**320-9.

274. **Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K. J. Tomaselli, K. M. Debatin, P. H. Krammer, and M. E. Peter.** 1998. Two CD95 (APO-1/Fas) signaling pathways. Embo J **17:**1675-87.

275. **Schendel, S. L., M. Montal, and J. C. Reed.** 1998. Bcl-2 family proteins as ion-channels. Cell Death Differ **5:**372-80.

276. **Schendel, S. L., Z. Xie, M. O. Montal, S. Matsuyama, M. Montal, and J. C. Reed.** 1997. Channel formation by antiapoptotic protein Bcl-2. Proc Natl Acad Sci U S A **94:**5113-8.

277. **Schinzel, A., T. Kaufmann, and C. Borner.** 2004. Bcl-2 family members: integrators of survival and death signals in physiology and pathology [corrected]. Biochim Biophys Acta **1644:**95-105.

278. **Schinzel, A., T. Kaufmann, M. Schuler, J. Martinalbo, D. Grubb, and C. Borner.** 2004. Conformational control of Bax localization and apoptotic activity by Pro168. J Cell Biol **164:**1021-32.

279. **Schlesinger, P. H., A. Gross, X. M. Yin, K. Yamamoto, M. Saito, G. Waksman, and S. J. Korsmeyer.** 1997. Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc Natl Acad Sci U S A **94:**11357-62.

280. **Schmelz, M., B. Sodeik, M. Ericsson, E. J. Wolffe, H. Shida, G. Hiller, and G. Griffiths.** 1994. Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. J Virol **68:**130-47.

281. **Schreiber, M., and G. McFadden.** 1994. The myxoma virus TNF-receptor homologue (T2) inhibits tumor necrosis factor-alpha in a species-specific fashion. Virology **204:**692-705.

282. **Schreiber, M., K. Rajarathnam, and G. McFadden.** 1996. Myxoma virus T2 protein, a tumor necrosis factor (TNF) receptor homolog, is secreted as a monomer and dimer that each bind rabbit TNFalpha, but the dimer is a more potent TNF inhibitor. J Biol Chem **271:**13333-41.

283. **Scorrano, L., M. Ashiya, K. Buttle, S. Weiler, S. A. Oakes, C. A. Mannella, and S. J. Korsmeyer.** 2002. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell **2:**55-67.

284. **Scorrano, L., and S. J. Korsmeyer.** 2003. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun **304:**437-44.

285. **Scott, F. L., J. B. Denault, S. J. Riedl, H. Shin, M. Renatus, and G. S. Salvesen.** 2005. XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. Embo J **24:**645-55.

286. **Seet, B. T., J. B. Johnston, C. R. Brunetti, J. W. Barrett, H. Everett, C. Cameron, J. Sypula, S. H. Nazarian, A. Lucas, and G. McFadden.** 2003. Poxviruses and immune evasion. Annu Rev Immunol **21:**377-423.

287. **Shi, Y.** 2002. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell **9:**459-70.

288. **Shimizu, S., Y. Eguchi, W. Kamiike, S. Waguri, Y. Uchiyama, H. Matsuda, and Y. Tsujimoto.** 1996. Retardation of chemical hypoxia-induced necrotic cell death by Bcl-2 and ICE inhibitors: possible involvement of common mediators in apoptotic and necrotic signal transductions. Oncogene **12:**2045-50.

289. **Shimizu, S., T. Ide, T. Yanagida, and Y. Tsujimoto.** 2000. Electrophysiological study of a novel large pore formed by Bax and the voltage-dependent anion channel that is permeable to cytochrome c. J Biol Chem **275:**12321-5.

290. **Shimizu, S., A. Konishi, T. Kodama, and Y. Tsujimoto.** 2000. BH4 domain of antiapoptotic Bcl-2 family members closes voltage- dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. Proc Natl Acad Sci U S A **97:**3100-5.

291. **Shimizu, S., M. Narita, and Y. Tsujimoto.** 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature **399:**483-7.

292. **Shimizu, S., and Y. Tsujimoto.** 2000. Proapoptotic BH3-only Bcl-2 family members induce cytochrome c release, but not mitochondrial membrane potential loss, and do not directly modulate voltage-dependent anion channel activity. Proc Natl Acad Sci U S A **97:**577-82.

293. **Shintani, T., and D. J. Klionsky.** 2004. Autophagy in health and disease: a double-edged sword. Science **306:**990-5.

294. **Shiozaki, E. N., J. Chai, D. J. Rigotti, S. J. Riedl, P. Li, S. M. Srinivasula, E. S. Alnemri, R. Fairman, and Y. Shi.** 2003. Mechanism of XIAP-mediated inhibition of caspase-9. Mol Cell **11:**519-27.

295. **Shisler, J., C. Yang, B. Walter, C. F. Ware, and L. R. Gooding.** 1997. The adenovirus E3-10.4K/14.5K complex mediates loss of cell surface Fas (CD95) and resistance to Fas-induced apoptosis. J Virol **71:**8299-306.

296. **Shisler, J. L., and B. Moss.** 2001. Molluscum contagiosum virus inhibitors of apoptosis: The MC159 v-FLIP protein blocks Fas-induced activation of procaspases and degradation of the related MC160 protein. Virology **282:**14-25.

297. **Skaletskaya, A., L. M. Bartle, T. Chittenden, A. L. McCormick, E. S. Mocarski, and V. S. Goldmacher.** 2001. A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proc Natl Acad Sci U S A **98:**7829-34.

298. **Smadel, J. E., and C. L. Hoagland.** 1942. Elementary bodies of vaccinia virus. Bacteriol. Rev. **6:**79-110.

299. **Smith, C. A., T. Davis, D. Anderson, L. Solam, M. P. Beckmann, R. Jerzy, S. K. Dower, D. Cosman, and R. G. Goodwin.** 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science **248:**1019-23.

300. **Smith, C. A., T. Davis, J. M. Wignall, W. S. Din, T. Farrah, C. Upton, G. McFadden, and R. G. Goodwin.** 1991. T2 open reading frame from the Shope fibroma virus encodes a soluble form of the TNF receptor. Biochem Biophys Res Commun **176:**335-42.

301. **Smith, C. A., F. Q. Hu, T. D. Smith, C. L. Richards, P. Smolak, R. G. Goodwin, and D. J. Pickup.** 1996. Cowpox virus genome encodes a second soluble homologue of cellular TNF receptors, distinct from CrmB, that binds TNF but not LT alpha. Virology **223:**132-47.

302. **Smith, G. L., A. Vanderplasschen, and M. Law.** 2002. The formation and function of extracellular enveloped vaccinia virus. J Gen Virol **83:**2915-31.

303. **Soloski, M. J., and J. A. Holowczak.** 1981. Characterization of supercoiled nucleoprotein complexes released from detergent-treated vaccinia virions. J Virol **37:**770-83.

304. **Srinivasula, S. M., M. Ahmad, T. Fernandes-Alnemri, G. Litwack, and E. S. Alnemri.** 1996. Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. Proc Natl Acad Sci U S A **93:**14486-91.

305. **Srinivasula, S. M., R. Hegde, A. Saleh, P. Datta, E. Shiozaki, J. Chai, R. A. Lee, P. D. Robbins, T. Fernandes-Alnemri, Y. Shi, and E. S. Alnemri.** 2001. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature **410:**112-6.

306. **Stennicke, H. R., Q. L. Deveraux, E. W. Humke, J. C. Reed, V. M. Dixit, and G. S. Salvesen.** 1999. Caspase-9 can be activated without proteolytic processing. J Biol Chem **274:**8359-62.

307. **Stewart, A. R., A. E. Tollefson, P. Krajcsi, S. P. Yei, and W. S. Wold.** 1995. The adenovirus E3 10.4K and 14.5K proteins, which function to prevent cytolysis by tumor necrosis factor and to down-regulate the epidermal growth factor receptor, are localized in the plasma membrane. J Virol **69:**172-81.

308. **Suda, T., T. Takahashi, P. Golstein, and S. Nagata.** 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell **75:**1169-78.

309. **Sun, C., M. Cai, R. P. Meadows, N. Xu, A. H. Gunasekera, J. Herrmann, J. C. Wu, and S. W. Fesik.** 2000. NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP. J Biol Chem **275:**33777-81.

310. **Sundararajan, R., A. Cuconati, D. Nelson, and E. White.** 2001. Tumor necrosis factor-alpha induces Bax-Bak interaction and apoptosis, which is inhibited by adenovirus E1B 19K. J Biol Chem **276:**45120-7.

311. **Sundararajan, R., and E. White.** 2001. E1B 19K blocks Bax oligomerization and tumor necrosis factor alpha-mediated apoptosis. J Virol **75:**7506-16.

312. **Susin, S. A., H. K. Lorenzo, N. Zamzami, I. Marzo, B. E. Snow, G. M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D. R. Goodlett, R. Aebersold, D. P. Siderovski, J. M. Penninger, and G. Kroemer.** 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature **397:**441-6.

313. **Susin, S. A., N. Zamzami, M. Castedo, T. Hirsch, P. Marchetti, A. Macho, E. Daugas, M. Geuskens, and G. Kroemer.** 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J Exp Med **184:**1331-41.

314. **Suzuki, M., R. J. Youle, and N. Tjandra.** 2000. Structure of Bax: coregulation of dimer formation and intracellular localization. Cell **103:**645-54.

315. **Suzuki, Y., Y. Imai, H. Nakayama, K. Takahashi, K. Takio, and R. Takahashi.** 2001. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell **8:**613-21.

316. **Symons, J. A., D. C. Tscharke, N. Price, and G. L. Smith.** 2002. A study of the vaccinia virus interferon-gamma receptor and its contribution to virus virulence. J Gen Virol **83:**1953-64.

317. **Talloczy, Z., W. Jiang, H. W. t. Virgin, D. A. Leib, D. Scheuner, R. J. Kaufman, E. L. Eskelinen, and B. Levine.** 2002. Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. Proc Natl Acad Sci U S A **99:**190-5.

318. **Tartaglia, L. A., T. M. Ayres, G. H. Wong, and D. V. Goeddel.** 1993. A novel domain within the 55 kd TNF receptor signals cell death. Cell **74:**845-53.

319. **Theodorakis, P., C. D'Sa-Eipper, T. Subramanian, and G. Chinnadurai.** 1996. Unmasking of a proliferation-restraining activity of the anti-apoptosis protein EBV BHRF1. Oncogene **12:**1707-13.

320. **Thome, M., P. Schneider, K. Hofmann, H. Fickenscher, E. Meinl, F. Neipel, C. Mattmann, K. Burns, J. L. Bodmer, M. Schroter, C. Scaffidi, P. H. Krammer, M. E. Peter, and J. Tschopp.** 1997. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature **386:**517-21.

321. **Thornberry, N. A., and Y. Lazebnik.** 1998. Caspases: enemies within. Science **281:**1312-6.

322. **Thornberry, N. A., T. A. Rano, E. P. Peterson, D. M. Rasper, T. Timkey, M. Garcia-Calvo, V. M. Houtzager, P. A. Nordstrom, S. Roy, J. P. Vaillancourt, K. T. Chapman, and D. W. Nicholson.** 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem **272:**17907-11.

323. **Tollefson, A. E., T. W. Hermiston, D. L. Lichtenstein, C. F. Colle, R. A. Tripp, T. Dimitrov, K. Toth, C. E. Wells, P. C. Doherty, and W. S. Wold.** 1998. Forced degradation of Fas inhibits apoptosis in adenovirus-infected cells. Nature **392:**726-30.

324. **Tollefson, A. E., K. Toth, K. Doronin, M. Kuppuswamy, O. A. Doronina, D. L. Lichtenstein, T. W. Hermiston, C. A. Smith, and W. S. Wold.** 2001. Inhibition of TRAIL-induced apoptosis and forced internalization of TRAIL receptor 1 by adenovirus proteins. J Virol **75:**8875-87.

325. **Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh.** 2000. Viral subversion of the immune system. Annu Rev Immunol **18:**861-926.

326. **Trapani, J. A., and M. J. Smyth.** 1993. Killing by cytotoxic T cells and natural killer cells: multiple granule serine proteases as initiators of DNA fragmentation. Immunol Cell Biol **71 (Pt 3):**201-8.

327. **Tsukumo, S. I., and S. Yonehara.** 1999. Requirement of cooperative functions of two repeated death effector domains in caspase-8 and in MC159 for induction and inhibition of apoptosis, respectively. Genes Cells **4:**541-9.

328. **Tsuruhara, T.** 1971. Immature particle formation of Yaba poxvirus studied by electron microscopy. J Natl Cancer Inst **47:**549-54.

329. **Tulman, E. R., C. L. Afonso, Z. Lu, L. Zsak, G. F. Kutish, and D. L. Rock.** 2004. The genome of canarypox virus. J Virol **78:**353-66.

330. **Turner, P. C., M. C. Sancho, S. R. Thoennes, A. Caputo, R. C. Bleackley, and R. W. Moyer.** 1999. Myxoma virus Serp2 is a weak inhibitor of granzyme B and interleukin- 1beta-converting enzyme in vitro and unlike CrmA cannot block apoptosis in cowpox virus-infected cells. J Virol **73:**6394-404.

331. **Turner, S. J., J. Silke, B. Kenshole, and J. Ruby.** 2000. Characterization of the ectromelia virus serpin, SPI-2. J Gen Virol **81:**2425-30.

332. **Upton, C., J. L. Macen, M. Schreiber, and G. McFadden.** 1991. Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. Virology **184:**370-82.

333. **Upton, C., S. Slack, A. L. Hunter, A. Ehlers, and R. L. Roper.** 2003. Poxvirus orthologous clusters: toward defining the minimum essential poxvirus genome. J Virol **77:**7590-600.

334. **Uren, A. G., E. J. Coulson, and D. L. Vaux.** 1998. Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. Trends Biochem Sci **23:**159-62.

335. **Uren, R. T., G. Dewson, C. Bonzon, T. Lithgow, D. D. Newmeyer, and R. M. Kluck.** 2005. Mitochondrial release of pro-apoptotic proteins: electrostatic interactions can hold cytochrome c but not Smac/DIABLO to mitochondrial membranes. J Biol Chem **280:**2266-74.

336. **Van Loo, G., H. Demol, M. van Gurp, B. Hoorelbeke, P. Schotte, R. Beyaert, B. Zhivotovsky, K. Gevaert, W. Declercq, J. Vandekerckhove, and P. Vandenabeele.** 2002. A matrix-assisted laser desorption ionization post-source decay (MALDI-PSD) analysis of proteins released from isolated liver mitochondria treated with recombinant truncated Bid. Cell Death Differ **9:**301-8.

337. **Vanderplasschen, A., M. Hollinshead, and G. L. Smith.** 1997. Antibodies against vaccinia virus do not neutralize extracellular enveloped virus but prevent virus release from infected cells and comet formation. J Gen Virol **78 (Pt 8):**2041-8.

338. **Vanderplasschen, A., M. Hollinshead, and G. L. Smith.** 1998. Intracellular and extracellular vaccinia virions enter cells by different mechanisms. J Gen Virol **79 (Pt 4):**877-87.

339. **Vanderplasschen, A., and G. L. Smith.** 1997. A novel virus binding assay using confocal microscopy: demonstration that the intracellular and extracellular vaccinia virions bind to different cellular receptors. J Virol **71:**4032-41.

340. **Vaux, D. L., S. Cory, and J. M. Adams.** 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature **335:**440-2.

341. **Vaux, D. L., and J. Silke.** 2003. Mammalian mitochondrial IAP binding proteins. Biochem Biophys Res Commun **304:**499-504.

342. **Vayssiere, J. L., P. X. Petit, Y. Risler, and B. Mignotte.** 1994. Commitment to apoptosis is associated with changes in mitochondrial biogenesis and activity in cell lines conditionally immortalized with simian virus 40. Proc Natl Acad Sci U S A **91:**11752-6.

343. **Verhagen, A. M., P. G. Ekert, M. Pakusch, J. Silke, L. M. Connolly, G. E. Reid, R. L. Moritz, R. J. Simpson, and D. L. Vaux.** 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell **102:**43-53.

344. **Verhagen, A. M., J. Silke, P. G. Ekert, M. Pakusch, H. Kaufmann, L. M. Connolly, C. L. Day, A. Tikoo, R. Burke, C. Wrobel, R. L. Moritz, R. J. Simpson, and D. L. Vaux.** 2002. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. J Biol Chem **277:**445-54.

345. **Verrier, F., B. Mignotte, G. Jan, and C. Brenner.** 2003. Study of PTPC composition during apoptosis for identification of viral protein target. Ann N Y Acad Sci **1010:**126-42.

346. **Vieira, H. L., A. S. Belzacq, D. Haouzi, F. Bernassola, I. Cohen, E. Jacotot, K. F. Ferri, C. El Hamel, L. M. Bartle, G. Melino, C. Brenner, V. Goldmacher, and G. Kroemer.** 2001. The adenine nucleotide translocator: a target of nitric oxide, peroxynitrite, and 4-hydroxynonenal. Oncogene **20:**4305-16.

347. **Vucic, D., W. J. Kaiser, and L. K. Miller.** 1998. A mutational analysis of the baculovirus inhibitor of apoptosis Op-IAP. J Biol Chem **273:**33915-21.

348. **Wajant, H., K. Pfizenmaier, and P. Scheurich.** 2003. Non-apoptotic Fas signaling. Cytokine Growth Factor Rev **14:**53-66.

349. **Wang, G., J. W. Barrett, S. H. Nazarian, H. Everett, X. Gao, C. Bleackley, K. Colwill, M. F. Moran, and G. McFadden.** 2004. Myxoma virus M11L prevents apoptosis through constitutive interaction with Bak. J Virol **78:**7097-111.

350. **Wang, G. H., J. Bertin, Y. Wang, D. A. Martin, J. Wang, K. J. Tomaselli, R. C. Armstrong, and J. I. Cohen.** 1997. Bovine herpesvirus 4 BORFE2 protein inhibits Fas- and tumor necrosis factor receptor 1-induced apoptosis and contains death effector domains shared with other gamma-2 herpesviruses. J Virol **71:**8928-32.

351. **Wang, H. W., T. V. Sharp, A. Koumi, G. Koentges, and C. Boshoff.** 2002. Characterization of an anti-apoptotic glycoprotein encoded by Kaposi's sarcoma-associated herpesvirus which resembles a spliced variant of human survivin. Embo J **21:**2602-15.

352. **Wang, X.** 2001. The expanding role of mitochondria in apoptosis. Genes Dev **15:**2922-33.

353. **Ware, C. F., P. D. Crowe, M. H. Grayson, M. J. Androlewicz, and J. L. Browning.** 1992. Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells. J Immunol **149:**3881-8.

354. **Wei, M. C., T. Lindsten, V. K. Mootha, S. Weiler, A. Gross, M. Ashiya, C. B. Thompson, and S. J. Korsmeyer.** 2000. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev **14:**2060-71.

355. **Wei, M. C., W. X. Zong, E. H. Cheng, T. Lindsten, V. Panoutsakopoulou, A. J. Ross, K. A. Roth, G. R. MacGregor, C. B. Thompson, and S. J. Korsmeyer.** 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science **292:**727-30.

356. **Weng, C., Y. Li, D. Xu, Y. Shi, and H. Tang.** 2005. Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in jurkat leukemia T cells. J Biol Chem **280:**10491-500.

357. **White, E., P. Sabbatini, M. Debbas, W. S. Wold, D. I. Kusher, and L. R. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. Mol Cell Biol **12:**2570-80.

358. **Widlak, P., L. Y. Li, X. Wang, and W. T. Garrard.** 2001. Action of recombinant human apoptotic endonuclease G on naked DNA and chromatin substrates: cooperation with exonuclease and DNase I. J Biol Chem **276:**48404-9.

359. **Wiens, M., A. Krasko, C. I. Muller, and W. E. Muller.** 2000. Molecular evolution of apoptotic pathways: cloning of key domains from sponges (Bcl-2 homology domains and death domains) and their phylogenetic relationships. J Mol Evol **50:**520-31.

360. **Wiens, M., A. Krasko, S. Perovic, and W. E. Muller.** 2003. Caspase-mediated apoptosis in sponges: cloning and function of the phylogenetic oldest apoptotic proteases from Metazoa. Biochim Biophys Acta **1593:**179-89.

361. **Wilkinson, J. C., A. S. Wilkinson, F. L. Scott, R. A. Csomos, G. S. Salvesen, and C. S. Duckett.** 2004. Neutralization of Smac/Diablo by inhibitors of apoptosis (IAPs). A caspase-independent mechanism for apoptotic inhibition. J Biol Chem **279:**51082-90.

362. **Willis, S. N., L. Chen, G. Dewson, A. Wei, E. Naik, J. I. Fletcher, J. M. Adams, and D. C. Huang.** 2005. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev **19:**1294-305.

363. **Wolter, K. G., Y. T. Hsu, C. L. Smith, A. Nechushtan, X. G. Xi, and R. J. Youle.** 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol **139:**1281-92.

364. **Wright, C. W., J. C. Means, T. Penabaz, and R. J. Clem.** 2005. The baculovirus anti-apoptotic protein Op-IAP does not inhibit Drosophila caspases or apoptosis in Drosophila S2 cells and instead sensitizes S2 cells to virus-induced apoptosis. Virology **335:**61-71.

365. **Xue, D., and H. R. Horvitz.** 1995. Inhibition of the Caenorhabditis elegans cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. Nature **377:**248-51.

366. **Xue, D., S. Shaham, and H. R. Horvitz.** 1996. The Caenorhabditis elegans cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. Genes Dev **10:**1073-83.

367. **Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz.** 1993. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell **75:**641-52.

368. **Yuan, J. Y., and H. R. Horvitz.** 1990. The Caenorhabditis elegans genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. Dev Biol **138:**33-41.

369. **Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J. L. Vayssiere, P. X. Petit, and G. Kroemer.** 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med **181:**1661-72.

370. **Zanardi, T. A., S. Yei, D. L. Lichtenstein, A. E. Tollefson, and W. S. Wold.** 2003. Distinct domains in the adenovirus E3 RIDalpha protein are required for degradation of Fas and the epidermal growth factor receptor. J Virol **77:**11685-96.

371. **Zhang, L., S. Shimizu, K. Sakamaki, S. Yonehara, and Y. Tsujimoto.** 2004. A caspase-8-independent signaling pathway activated by Fas ligation leads to exposure of the Bak N terminus. J Biol Chem **279:**33865-74.

372. **Zhou, Q., J. F. Krebs, S. J. Snipas, A. Price, E. S. Alnemri, K. J. Tomaselli, and G. S. Salvesen.** 1998. Interaction of the baculovirus anti-apoptotic protein p35 with caspases. Specificity, kinetics, and characterization of the caspase/p35 complex. Biochemistry **37:**10757-65.

373. **Zhou, Q., S. Snipas, K. Orth, M. Muzio, V. M. Dixit, and G. S. Salvesen.** 1997. Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J Biol Chem **272:**7797-800.

374. **Zong, W. X., D. Ditsworth, D. E. Bauer, Z. Q. Wang, and C. B. Thompson.** 2004. Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev **18:**1272-82.

375. **Zong, W. X., T. Lindsten, A. J. Ross, G. R. MacGregor, and C. B. Thompson.** 2001. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev **15:**1481-6.

376. **Zoog, S. J., J. J. Schiller, J. A. Wetter, N. Chejanovsky, and P. D. Friesen.** 2002. Baculovirus apoptotic suppressor P49 is a substrate inhibitor of initiator caspases resistant to P35 in vivo. Embo J **21:**5130-40.

377. **Zoratti, M., and I. Szabo.** 1995. The mitochondrial permeability transition. Biochim Biophys Acta **1241:**139-76.

378. **Zou, H., Y. Li, X. Liu, and X. Wang.** 1999. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem **274:**11549-56.

Chapter 2: Vaccinia Virus Infection Disarms the Mitochondrion-Mediated Pathway of the Apoptotic Cascade by Modulating the Permeability Transition Pore

Preface

The data presented in all figures was generated by myself with the exception of Figure 2-1 which was generated by Dr. Michele Barry. I wrote the first draft of the manuscript. A major editorial contribution from my supervisor, Dr. Michele Barry, led to the final version of the paper. A version of this chapter has been published. Wasilenko ST, Meyers AF, Vander Helm K, and Barry M. Journal of Virology, 2001, 75: 11437-48.

2.1 Introduction

An important function of the cell-mediated immune response is the detection and elimination of virus-infected cells as a means to arrest viral propagation. To do this, immune effector cells rely on the production of cytokines and the recognition of virus infected cells by cytotoxic T lymphocytes (CTL) to induce programmed cell death, or apoptosis (31). Apoptosis results in a variety of cellular changes including cell shrinkage, DNA fragmentation, chromatin condensation, and finally the formation of apoptotic bodies. These changes are mediated by biochemical events involving a family of cysteine proteases termed caspases (45, 61). Following an apoptotic stimulus, caspases become proteolytically activated and function to cleave cellular proteins, including other members of the caspase family. Recently, it has also become clear that within cells instructed to die, mitochondria play a central role in the execution of apoptosis (13). The induction of apoptosis results in both structural and physiological alterations to mitochondria, including disruption of electron transport and energy metabolism, production of reactive oxygen species, loss of the membrane potential, and release of proapoptotic proteins, including cytochrome c (37).

In order for a virus to replicate and disseminate within a host, manipulation of the apoptotic process is essential (2, 48, 64). To ensure their survival, viruses have evolved strategies that target crucial components within the apoptotic cascade. For example, virus-encoded inhibitors of apoptosis have been identified that either directly or indirectly modulate caspase activation. One of the best-studied viral caspase inhibitors is the cowpox virus-encoded cytokine response modifier A (CrmA), also known as Spi-2.

CrmA/Spi-2 inhibits both Fas- and tumor necrosis factor (TNF)-induced apoptosis via interaction with caspase 8 (33, 59, 71). In a similar manner, baculoviruses encode p35, a broad-spectrum caspase inhibitor that protects infected cells from apoptosis (8, 70). As well, baculoviruses and African swine fever virus modulate the activation and activity of caspases through the expression of inhibitors of apoptosis (IAPs) (11, 14, 46). In addition to modulating caspase activity, viruses have also developed strategies that interfere with other components of the death pathway. For example, poxviruses encode secreted TNF receptors that inhibit TNF-induced apoptosis by blocking ligand-receptor interactions (27, 52, 55). Gammaherpesviruses and the poxvirus molluscum contagiosum encode viral Fas-associated death domain-like interleukin-1-converting enzyme inhibitory proteins (vFLIPs), which interfere with recruitment of caspase 8 to the cytoplasmic domains of Fas and TNF receptor 1 (5, 28, 60). Additionally, adenovirus has evolved an elaborate scheme to stimulate the internalization of cell surface Fas (53, 62).

Since mitochondria play a central role in cell death, viruses have also established mechanisms to modulate the mitochondrial component of the apoptotic pathway. Members of the cellular Bcl-2 family influence the integrity of the mitochondria (10, 21), and many viruses encoding Bcl-2-like proteins have been identified. Viral Bcl-2 homologues with anti-apoptotic function have been found in adenovirus (65) and African swine fever virus (1, 7) as well as in members of the gammaherpesvirus family including Epstein-Barr virus, equine herpesvirus 2, herpesvirus saimiri, Kaposi's sarcoma-associated herpesvirus, bovine herpesvirus, herpesvirus ateles, alcelaphine herpesvirus 1, and murine gammaherpesvirus 68 (64). In addition, novel virus gene products that act at the mitochondrial checkpoint but lack homology to Bcl-2 have also been identified.

VMIA, encoded by human cytomegalovirus (HCMV), inhibits apoptosis and the release of cytochrome c in HeLa cells through interaction with the adenine nucleotide translocator (ANT) subunit of the permeability transition pore (PTP) (20). M11L, encoded by the rabbit-specific poxvirus myxoma virus, localizes to the mitochondria and inhibits staurosporine-induced loss of mitochondrial membrane potential and apoptosis (17).

The large number of viruses encoding proteins that function to maintain the integrity of the mitochondria led us to hypothesize that vaccinia virus, a member of the poxvirus family, would employ a mechanism to directly modulate the mitochondrial apoptotic pathway. To determine whether vaccinia virus infection could inhibit the mitochondrion-mediated apoptotic pathway, we monitored the ability of vaccinia virus strain Copenhagen, which is naturally devoid of the caspase 8 inhibitor CrmA/Spi2, to inhibit Fas- and staurosporine-mediated apoptosis. We show here, for the first time, that vaccinia virus modulates the apoptotic mitochondrial pathway by inhibiting the PTP, thereby preserving the mitochondrial membrane potential and retaining cytochrome c.

2.2 Materials and Methods

Cell and Viruses.

Jurkat cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (both from Gibco BRL Life Technologies, Inc.), 100 μM 2-mercaptoethanol, 50 U of penicillin/ml, and 50 μg of streptomycin/ml (RHFM). Stably transfected Jurkat cells were generated as previously described (3) and maintained in RHFM supplemented with 800 μg of G418/ml. Recombinant vaccinia virus strain

Copenhagen expressing beta-galactosidase (VV65) was a gift from G. McFadden (Robarts Research Institute, London, Ontario, Canada) (26). VV65 was routinely propagated in baby green monkey kidney (BGMK) cells, a gift from S. Dales, and grown in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum (both from Gibco BRL Life Technologies, Inc.), 50 U of penicillin/ml, 50 µg of streptomycin/ml, and 2 mM glutamine. Viruses were isolated as previously described (57).

Virus Infection.

Jurkat cells were infected at a multiplicity of infection (MOI) of 10 PFU per cell in 200 µl of RHFM at 37°C. After 1 h, the cells were supplemented with additional RHFM for 4 h and incubated at 37°C under 5% before induction of apoptosis. The efficiency of virus infection was routinely quantified by colorimetric analysis using the *lacZ* gene. In all experiments, the efficiency of infection was found to be greater than 95%. When necessary, VV65 was UV inactivated for 60 minutes prior to infection and cytosine arabinoside (Sigma Chemical Co.) was added to a final concentration of 40 µg/ml.

Antibodies.

The p20 fragment of caspase 3 (C3p20) was amplified by PCR from pSKII:CPP32 using the forward oligonucleotide 5'-GGATCCTCTGGAATATCCCT GGAC-3' containing a BamHI restriction site and the reverse oligonucleotide 5'-

GTCGACGTCTGTCTCAATGCCACA-3' containing a SalI restriction site. Amplified C3p20 was subcloned into pGex4T-3 (Pharmacia Biotech) to construct pGex4T-3:C3p20. pGex4T-3-C3p20 was transformed into BL21DE3, and protein expression was induced by the addition of 0.1 mM isopropyl-D-thiogalactopyranoside (IPTG) (Rose Scientific Ltd.). Glutathione S-transferase (GST)-C3p20 was purified utilizing glutathione Sepharose 4B according to the manufacturer's instructions (Pharmacia Biotech). Pet15b-Bid, a gift from X. Wang (University of Texas Southwestern Medical Center, Dallas, Tex.) was used to express His-tagged Bid (40). Recombinant His-tagged Bid was purified using a Ni^{+2} column according to the manufacturer's instructions (Novagen). Rabbits were immunized by injection of 500μg of bacterially expressed GST-caspase 3 or His-Bid in Freund's complete adjuvant (Gibco BRL Life Technologies). At monthly intervals the animals were boosted with 500μg of antigen in Freund's incompleteadjuvant (Gibco BRL Life Technologies), and antiserum was collected 10 days after the fourth boost. The anti-cytochrome c antibody (clone 7H8.2Cl2) was purchased from PharMingen. Anti-human Fas immunoglobulin M (IgM) (clone CH11) was purchased from Upstate Biotechnology. Goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated antibodies were purchased from Bio-Rad and used at dilutions of 1:3,000 and 1:10,000, respectively.

Apoptosis Induction.

Cells were induced to undergo apoptosis by addition of either 250ng of activating anti-Fas/ml or 1 to 5μM staurosporine (Sigma Chemical Co.) as outlined in the results.

Chromium Release Assay.

^{51}Cr release assays were performed as previously described (4). Briefly, cells were labeled with 100μCi of ^{51}Cr at 37°C for 1 h. Labeled target cells were incubated with 250ng of anti-Fas clone CH11/ml, and ^{51}Cr release was quantitated after 8 h. ^{51}Cr release was calculated by the following equation: percent lysis = 100 × (sample release − spontaneous release)/(total release − spontaneous release). Standard deviations were generated from three replicates.

Cytochrome c Release Assay.

Cytochrome c release was monitored as previously described (25, 49). Following apoptosis treatment, 2×10^6 or 5×10^6 Jurkat cells were permeabilized by incubation in digitonin lysis buffer containing 75mM NaCl, 1mM NaH_2PO_4, 8mM Na_2HPO_4, 250mM sucrose, and 190μg of digitonin (Sigma Chemical Co.)/ml. Cells were incubated on ice for 10 minutes, after which the mitochondria-containing pellet and the cytosolic supernatant were separated by centrifugation at 10,000×g for 5 minutes. Mitochondrial pellets were resuspended in 0.1% Triton X-100-25mM Tris (pH 8.0) prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Measurement of Mitochondrial Membrane Potential.

Changes in mitochondrial membrane potential were quantified by staining cells with tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) (16, 18, 43). Cells were loaded with TMRE by a 30 minute incubation (at 37°C, under 5% CO_2) in RHFM containing 0.2μM TMRE. As a control, cells were also treated with a membrane uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (ClCCP) (Sigma Chemical Co.), at a final concentration of 50μM, for 15 minutes at 37°C under 5% CO_2. To trigger the permeability transition, cells were treated with either 1 μM staurosporine or 300μM *t*-butylhydroperoxide (both from Sigma Chemical Co.) for 1 or 2 hours, respectively. Prior to flow cytometric analysis, cells were washed with phosphate-buffered saline (PBS) containing 1% fetal bovine serum. TMRE fluorescence was acquired through the FL-2 channel equipped with a 585nm filter (band pass, 42nm). Data were acquired on either 10,000 or 20,000 cells per sample with fluorescence signals at logarithmic gain. Data were analyzed with CellQuest software, and standard deviations were generated from three independent experiments.

Detection of DNA Fragmentation.

DNA fragmentation was assessed using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) kit (Roche Diagnostics Co.). Briefly, cells were harvested, washed in PBS containing 1% fetal calf serum, fixed in 2% paraformaldehyde, and permeabilized in 0.1% Triton X-100. Fixed and permeabilized cells were incubated for 1 h at 37°C in a solution containing 25mM Tris (pH 6.6), 200mM cacodylate, 1mM $CoCl_2$, 0.6nM fluorescein-12-dUTP, and 25U of terminal deoxynucleotidyltransferase (Roche Diagnostics Co.). Analysis was performed on a Becton Dickinson FACScan equipped with an argon laser at 15mV with an excitation wavelength of 488nm. Emission wavelengths were detected through the FL-1 channel equipped with a 530nm filter (band pass, 20nm). Data was acquired on 10,000 cells per sample with light scatter signals at linear gain and fluorescence signals at logarithmic gain.

***In vitro* Reconstitution Assay.**

Mitochondria were purified as previously described (24, 49). For each test sample, 4×10^7 cells were washed in buffer A containing 20mM morpholinepropanesulfonic acid (MOPS; pH 7.4), 100mM sucrose, and 1mM EGTA. Cells were resuspended in buffer B containing 20mM MOPS (pH 7.4), 100mM sucrose, 1mM EGTA, 5% Percoll (Sigma Chemical Co.), and 190µg of digitonin/ml. Following a 15 minute incubation on ice with intermittent inversion, nuclei were pelleted at 2,500×g for 10 minutes at 4°C. The pellet was discarded, and the supernatant was centrifuged at 15,000×g for 15 minutes at 4°C. The mitochondrial fraction was collected and washed three times in buffer A and resuspended in buffer C containing 20mM MOPS (pH 7.4), 300mM sucrose, and 1.0mM EGTA. The protein concentration of the mitochondrial fraction was determined using the bicinchoninic acid (BCA) kit from Pierce Chemical Company. For the in vitro assay, 6µg of purified mitochondria was either incubated with 2, 5, or 10ng of recombinant Bid in the presence or absence of 0.25µg of purified granzyme B or treated with 5, 10, or 15mM atractyloside (Sigma Chemical Co.) for 40 minutes at 37°C. Granzyme B was purified from YT-Indy cells as previously described (9, 23). Following the addition of granzyme B and His-tagged Bid, the samples were incubated at 37°C for 60 minutes. Samples were then centrifuged at 15,000×g to separate the mitochondrial pellet from the supernatant prior to SDS-PAGE analysis.

Immunoblotting.

Cellular lysates were analyzed by electrophoresis on an SDS-15% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Osmonics Inc.) using a semidry transfer apparatus (Tyler Research Instruments) for 2.5 h at 500 mA. Membranes were blocked for at least 3 h in PBS containing 0.1% Tween and 5% skim milk. Caspase 3 and Bid were detected using polyclonal rabbit anti-caspase 3 and anti-Bid at a dilution of 1:10,000. Cytochrome c was detected using a monoclonal antibody at a 1:1,000 dilution. All primary antibodies were incubated with the membranes overnight at 4°C. Membranes were probed with either a goat anti-mouse (1:3,000) or a goat anti-rabbit (1:10,000) horseradish peroxidase-conjugated antibody. Proteins were visualized with a chemiluminescence detection system according to the manufacturer's directions (Amersham Pharmacia Biotech).

2.3 Results

Vaccinia Virus strain Copenhagen-infected Cells are Resistant to anti-Fas-mediated Apoptosis.

To determine whether vaccinia virus infection could inhibit the mitochondrion-mediated apoptotic pathway, we monitored the ability of vaccinia virus strain Copenhagen to inhibit anti-Fas-induced apoptosis of Jurkat cells. We utilized Jurkat cells, since Fas-induced apoptosis occurs exclusively via the mitochondrial pathway in this cell line (51). In addition, we utilized a strain of vaccinia virus, strain Copenhagen, which is naturally devoid of the caspase 8 inhibitor CrmA/Spi-2 (19). Jurkat cells were either mock infected or infected with vaccinia virus strain Copenhagen. At 5 h postinfection, apoptosis was triggered by the addition of anti-Fas and cell death was measured by ^{51}Cr release. As shown in Figure 2-1, mock-infected cells treated with anti-Fas displayed approximately 30% ^{51}Cr release. This release was completely inhibited by pretreating the cells with the broad-spectrum caspase inhibitor zVAD.fmk, indicating that cytolysis was directly dependent on caspase activation. Infection of Jurkat cells with vaccinia virus strain Western Reserve, which encodes a functional Spi-2, drastically reduced the levels of ^{51}Cr released. As expected, Jurkat cells stably transfected with either Spi-2 or Bcl-2 were protected from anti-Fas-triggered death. Most significantly, Jurkat cells infected with vaccinia virus strain Copenhagen, lacking a functional CrmA/Spi-2, also inhibited death mediated via the Fas pathway, clearly suggesting that vaccinia virus strain Copenhagen employs an additional anti-apoptotic mechanism.

To begin to determine the point at which vaccinia virus strain Copenhagen-infected Jurkat cells were resistant to cell death, we monitored anti-Fas-mediated caspase 3 activation. Caspase 3 activation was assessed by western blot analysis using an antibody raised against the large subunit of the active caspase. Over 8 h, uninfected cells treated with anti-Fas showed processing of caspase 3 from the full-length 32-kDa procaspase to the mature 19- and 17-kDa forms (Figure 2-2A). In contrast, cells infected with vaccinia virus strain Copenhagen and treated with the anti-Fas antibody exhibited only minor amounts of active caspase 3 (Figure 2-2B), indicating that infected cells were

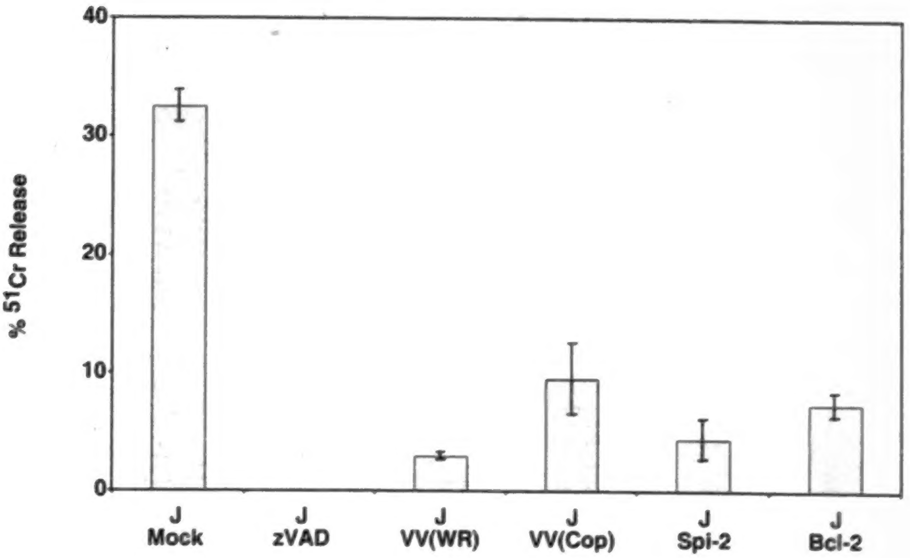


Figure 2-1 Vaccinia virus strain Copenhagen protects cells from anti-Fas-mediated death.
Jurkat cells were either mock infected or infected with either vaccinia virus strain Copenhagen or vaccinia virus strain Western Reserve at an MOI of 10. Following 5 h of infection, cells were treated with 250 ng of anti-Fas antibody/ml to induce apoptosis, and cell death was monitored 8 h later by ^{51}Cr release. As controls, Jurkat cells that overexpress SPI-2 and Bcl-2 were also treated with anti-Fas, and Jurkat cells were pretreated for 30 min with 100 μM zVAD.fmk prior to addition of anti-Fas. Standard deviations were generated from three replicates.

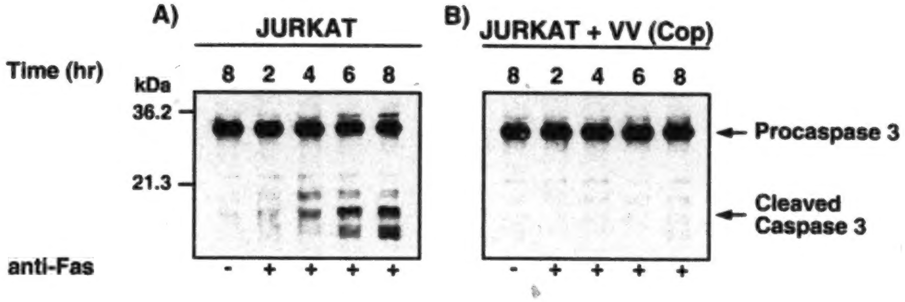

A)
JURKAT
B)
JURKAT + VV (Cop)
Time (hr)
kDa
8 2 4 6 8
8 2 4 6 8
36.2
21.3
Procaspase 3
Cleaved Caspase 3
anti-Fas
- + + + +
- + + + +

Figure 2-2 Vaccinia virus strain Copenhagen infection inhibits activation of caspase 3.

Jurkat cells were either mock infected or infected with vaccinia virus strain Copenhagen at an MOI of 10 and treated with 250 ng of anti-Fas antibody/ml for 2, 4, 6, or 8 h. At the times indicated, cells were permeabilized with digitonin, and caspase 3 processing was monitored by western blot analysis. (A) Mock-infected Jurkat cells; (B) Jurkat cells infected with vaccinia virus strain Copenhagen.

protected from apoptosis.

Since infection of Jurkat cells with vaccinia virus strain Copenhagen inhibited the activation of caspase 3, and since mitochondrial release of cytochrome c is necessary for caspase 3 activation, we assessed the ability of vaccinia virus strain Copenhagen to inhibit the release of cytochrome c following treatment with anti-Fas (13, 37, 39). To detect the release of cytochrome c, cells were fractionated into mitochondrial and cytosolic fractions and the release of cytochrome c was detected by western blot analysis. Using this approach, apoptotic extracts demonstrated the translocation of cytochrome c from the mitochondria to the cytosolic fraction (Figure 2-3A). Translocation of cytochrome c was first detected as early as 4 h following the addition of anti-Fas and was found to increase over time (Figure 2-3A). As a control, we also monitored cytochrome c release in Jurkat cells that overexpress the anti-apoptotic protein Bcl-2. As previously documented, Bcl-2 expression inhibited the translocation of cytochrome c to the cytosol (Figure 2-3B) (35, 66). Fas-mediated release of cytochrome c was also monitored in Jurkat cells infected with vaccinia virus strain Copenhagen. As shown in Figure 2-3C, infection with vaccinia virus strain Copenhagen significantly interfered with the release of cytochrome c, indicating that vaccinia virus strain Copenhagen inhibits the Fas-mediated apoptotic pathway upstream of cytochrome c release.

Vaccinia Virus strain Copenhagen Infection Inhibits Cytochrome c Release in Isolated Mitochondria but not Cleavage of Bid.

The ability of vaccinia virus strain Copenhagen to inhibit Fas-induced cytochrome c release suggested to us that the virus could potentially hinder apoptosis by interfering

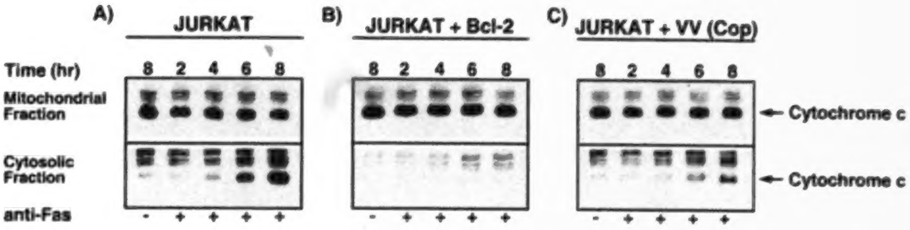

A)
JURKAT
B)
JURKAT + Bcl-2
C)
JURKAT + VV (Cop)
Time (hr)
8 2 4 6 8
8 2 4 6 8
8 2 4 6 8
Mitochondrial Fraction
Cytosolic Fraction
Cytochrome c
Cytochrome c
anti-Fas
- + + + +
- + + + +
- + + + +

Figure 2-3 Vaccinia virus strain Copenhagen inhibits cytochrome c translocation. Jurkat cells were either mock infected or infected with virus. Following infection, cells were treated with 250 ng of anti-Fas/ml for 2, 4, 6, or 8 h to induce cytochrome c translocation. At the times indicated, cells were permeabilized with digitonin and fractionated into the mitochondrion-containing membranous fraction and the soluble cytoplasmic fraction, and cytochrome c was assessed by western blot analysis. (A) Mock-infected Jurkat cells; (B) mock infected Jurkat cells that overexpress Bcl-2; (C) Jurkat cells infected with vaccinia virus strain Copenhagen.

with activation of the proapoptotic Bcl-2 family member Bid. During Fas-mediated apoptosis, Bid is cleaved by caspase 8, prompting the translocation of truncated Bid to the mitochondria, resulting in the release of cytochrome c (38, 40). Additionally, the serine proteinase granzyme B, which is released from activated CTL, is also able to induce apoptosis through the cleavage of Bid (3, 24). To investigate the potential inhibition of Bid activation by vaccinia virus strain Copenhagen, we performed an in vitro apoptotic reconstitution assay involving isolated mitochondria, recombinant Bid, and purified granzyme B. Mitochondria were purified from both mock-infected and virus-infected Jurkat cells as well as from Jurkat cells stably expressing Bcl-2. Isolated mitochondria were incubated with increasing amounts of recombinant Bid, either in the presence or in the absence of purified granzyme B, and proteolytic cleavage of Bid was monitored by western blot analysis. Figure 2-4A demonstrates that in the presence of purified mitochondria and granzyme B, recombinant Bid underwent proteolytic cleavage and activation in this assay. In agreement with previously published results, Bid was also processed in the presence of isolated mitochondria from cells overexpressing Bcl-2 (22, 44) (Figure 2-4B). The processing of Bid was found to be unaltered when mitochondria from vaccinia virus-infected cells were used in the same assay, indicating that vaccinia virus infection does not inhibit the proteolytic processing of Bid (Figure 2-4C).

Since vaccinia virus infection did not suppress granzyme B-induced Bid cleavage, we next asked if mitochondria isolated from vaccinia virus-infected cells could inhibit granzyme B-induced cytochrome c release in the presence of Bid. Mitochondria isolated from mock-infected Jurkat cells were incubated with increasing amounts of recombinant Bid in the presence of granzyme B, resulting in the translocation of cytochrome c from

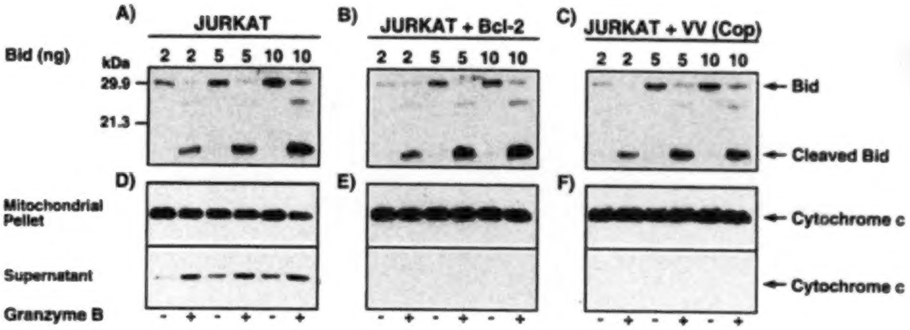

A)
JURKAT
B)
JURKAT + Bcl-2
C)
JURKAT + VV (Cop)
Bid (ng)
kDa
2 2 5 5 10 10
2 2 5 5 10 10
2 2 5 5 10 10
29.9
21.3
Bid
Cleaved Bid
D)
E)
F)
Mitochondrial Pellet
Cytochrome c
Supernatant
Cytochrome c
Granzyme B
- + - + - +
- + - + - +
- + - + - +

Figure 2-4 Vaccinia virus infection protects against granzyme B-mediated cytochrome c release from isolated mitochondria by a mechanism downstream of Bid activation.

Mitochondria were isolated from mock-infected Jurkat cells, Jurkat cells overexpressing Bcl-2, and Jurkat cells infected with vaccinia virus strain Copenhagen. Purified mitochondria were incubated for 60 min at 37°C with 2, 5, or 10 ng of recombinant Bid either in the presence or in the absence of granzyme B. Following treatment, samples were fractionated into mitochondria-containing and soluble fractions and the proteins were resolved by SDS-PAGE. (A through C) western blot analysis of Bid cleavage in mitochondria isolated either from mock-infected cells (A), from cells overexpressing Bcl-2 (B), or from vaccinia virus strain Copenhagen-infected cells (C). (D through F) western blot analysis of cytochrome c translocation from purified mitochondria to supernatant fractions in mitochondria isolated either from mock-infected cells (D), from mock-infected cells overexpressing Bcl-2 (E), or from vaccinia virus strain Copenhagen-infected cells (F).

the mitochondria to the supernatant (Figure 2-4D). As shown in Figure 2-4D, an increase in the amount of cytochrome c translocation was detected following the addition of granzyme B and increasing amounts of recombinant Bid. At 5 and 10 ng of Bid, we found that some cytochrome c translocation was independent of Bid cleavage, as previously reported (40, 67) (Figure 2-4D). The release of cytochrome c was completely abolished in mitochondria isolated from Jurkat cells overexpressing the anti-apoptotic protein Bcl-2 (Figure 2-4E). Inhibition of cytochrome c translocation was also seen in mitochondria isolated from vaccinia virus-infected cells (Figure 2-4F). Taken together, these results indicated that cytochrome c release was inhibited in mitochondria isolated from infected cells and suggested that vaccinia virus infection directly modulated the mitochondrial arm of the apoptotic pathway.

Vaccinia Virus strain Copenhagen-infected Cells are Resistant to Staurosporine-mediated Apoptosis.

In view of our findings demonstrating that vaccinia virus strain Copenhagen could inhibit Fas-mediated cytochrome c release and cytochrome c release from isolated mitochondria, we assessed the ability of vaccinia virus strain Copenhagen to directly inhibit the mitochondrial route to apoptotic death. To determine if vaccinia virus strain Copenhagen could directly inhibit the mitochondrial cascade, we treated cells with the proapoptotic reagent staurosporine, which triggers the mitochondrion-mediated apoptotic pathway (6, 58). Mock-infected or virus-infected Jurkat cells were treated with staurosporine, and the levels of DNA fragmentation were measured using the TUNEL assay and flow cytometry. As shown in Figure 2-5, untreated cells demonstrated low levels of DNA fragmentation (Figure 2-5 a, d, and f). Upon staurosporine treatment, 42% of the mock-infected Jurkat cell population showed DNA fragmentation (Figure 2-5b). Preincubation with the broad-spectrum caspase inhibitor zVAD.fmk completely inhibited staurosporine-induced DNA fragmentation, clearly demonstrating that staurosporine-induced DNA fragmentation occurred via caspase activation, as expected (Figure 2-5c). In addition, stably transfected Jurkat cells that overexpress Bcl-2 were also found to be resistant to apoptosis, indicating that staurosporine-mediated cell death occurred via the mitochondrial pathway (Figure 2-5e). Most importantly, vaccinia virus strain Copenhagen-infected Jurkat cells treated with staurosporine displayed clear protection from apoptosis, with only 7% of the cells showing DNA fragmentation (Figure 2-5g). To determine if the block was upstream of caspase 3activation, cells were treated with staurosporine for 2, 4, or 6 hr and caspase 3 processing was monitored by western blot analysis. As shown in Figure 2-6A, mock-infected Jurkat cells treated with staurosporine displayed rapid conversion of the 32-kDa procaspase 3 to the active fragments. This conversion was significantly inhibited both in Bcl-2-overexpressing cells and in cells infected with vaccinia virus strain Copenhagen (Figure 2-6 B and C). Compared to mock-infected cells, vaccinia virus strain Copenhagen-infected Jurkat cells treated with staurosporine displayed drastic reductions in levels of the 19- and 17-kDa caspase 3 fragments and maintenance of the full-length 32-kDa proform, indicating that apoptosis inhibition occurred upstream of caspase 3 activation (Figure 2-6 A and B).

Since Jurkat cells infected with vaccinia virus strain Copenhagen and induced to undergo apoptosis with anti-Fas inhibited cytochrome c translocation from mitochondria

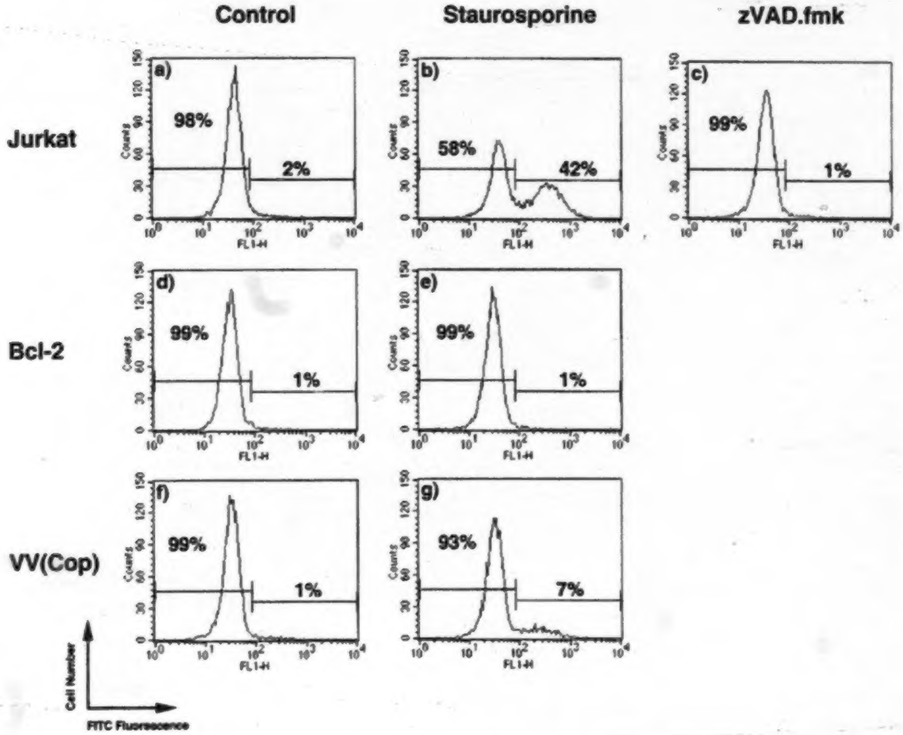


Figure 2-5 DNA fragmentation is blocked by vaccinia virus strain Copenhagen infection.

Jurkat cells were either mock infected or infected with vaccinia virus strain Copenhagen. Following infection, cells were treated with 2.5 μM staurosporine for 2 h, and DNA fragmentation was assessed by TUNEL as outlined in Materials and Methods. (a) Untreated Jurkat cells; (b) Jurkat cells treated with staurosporine; (c) Jurkat cells treated with staurosporine in the presence of 100 μM zVAD.fmk; (d) Jurkat cells overexpressing Bcl-2; (e) Jurkat cells overexpressing Bcl-2 treated with staurosporine; (f) Jurkat cells infected with vaccinia virus strain Copenhagen; (g) Jurkat cells infected with vaccinia virus and treated with staurosporine.

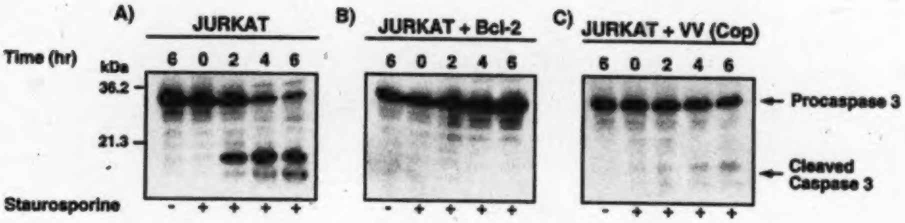

A)
JURKAT
B)
JURKAT + Bcl-2
C)
JURKAT + VV (Cop)
Time (hr)
kDa
6 0 2 4 6
6 0 2 4 6
6 0 2 4 6
36.2
21.3
Procaspase 3
Cleaved Caspase 3
Staurosporine
- + + + +
- + + + +
- + + + +

Figure 2-6 Staurosporine-induced caspase 3 activation is inhibited by vaccinia virus strain Copenhagen infection.

Jurkat cells were either mock infected or infected with virus; following infection, they were treated with 5 μM staurosporine for 0, 2, 4, or 6 h. At the times indicated, cells were permeabilized with digitonin and the proteins were resolved by SDS-PAGE. The activation of caspase 3 was monitored by western blot analysis. (A) Mock-infected Jurkat cells; (B) mock-infected Jurkat cells that overexpress Bcl-2; (C) Jurkat cells infected with vaccinia virus strain Copenhagen.

to the cytosol, we investigated the possibility that staurosporine-induced cytochrome c translocation would be inhibited by virus infection as well. Mock-infected cells treated with staurosporine exhibited a dramatic loss of cytochrome c from the mitochondrial fraction and subsequent accumulation in the cytoplasmic fraction (Figure 2-7A). In contrast to cells treated with anti-Fas antibody, cells treated with staurosporine displayed cytochrome c translocation as early as 2 hr post treatment, and we routinely detected complete translocation of cytochrome c to the cytoplasmic fraction after 6 h of treatment. As anticipated, staurosporine-induced translocation of cytochrome c was completely inhibited in Jurkat cells engineered to overexpress Bcl-2 (35, 66) (Figure 2-7B). Most importantly, in cells infected with vaccinia virus strain Copenhagen, cytochrome c release was drastically reduced (Figure 2-7C). In contrast to the situation in mock-infected cells, complete translocation of cytochrome c from the mitochondrial to the cytosolic fraction was inhibited by vaccinia virus infection, and cytosolic cytochrome c was only partially evident at 4 and 6 h post-staurosporine treatment.

Vaccinia Virus Infection Inhibits Disruption of the Mitochondrial Inner Membrane Potential and Opening of the PTP.

During apoptosis the release of cytochrome c coincides with loss of the inner mitochondrial membrane potential (41, 69). Disruption of the inner mitochondrial membrane potential is thought to occur due to the opening of the PTP (12, 36, 37). Since vaccinia virus strain Copenhagen infection inhibited cytochrome c translocation and apoptosis, we asked if vaccinia virus infection was able to inhibit apoptosis by

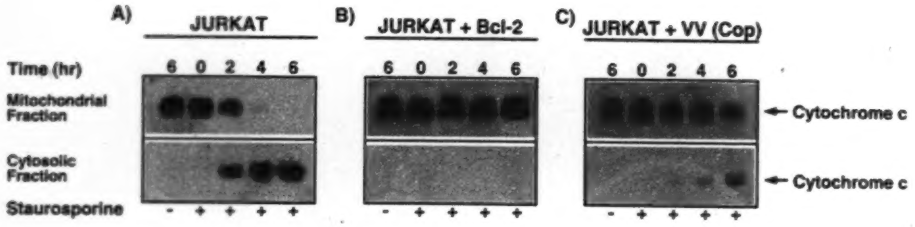


Figure 2-7 Staurosporine-induced cytochrome c release is inhibited by vaccinia virus strain Copenhagen infection.

Jurkat cells were either mock infected or infected with virus and were treated with 5 μM staurosporine for 0, 2, 4, or 6 h. At the times indicated, cells were fractionated into mitochondria-containing membrane fractions and cytoplasmic fractions by the addition of digitonin. The translocation of cytochrome c was monitored by western blot analysis. (A) Mock-infected Jurkat cells; (B) mock-infected Jurkat cells that overexpress Bcl-2; (C) Jurkat cells infected with vaccinia virus strain Copenhagen.

maintaining mitochondrial integrity and the inner mitochondrial membrane potential. Changes in the membrane potential were monitored by assaying the uptake of TMRE, a fluorescent mitochondrion-specific dye (18, 50). Disruption of the membrane potential in mock-infected and infected cells following staurosporine treatment was monitored by TMRE fluorescence. In untreated Jurkat cells, 94% of the cells demonstratedTMRE fluorescence, indicating an intact mitochondrial membrane potential (Figure 2-8a). Upon staurosporine treatment, 48% of the cells exhibited a reduction in TMRE fluorescence (Figure 2-8b). As a control, Jurkat cells were treated with a membrane uncoupler, ClCCP, resulting in the reduction of TMRE fluorescence in all cells (Figure 2-8c). Jurkat cells treated with staurosporine in the presence of the caspase inhibitor zVAD.fmk still demonstrated a loss in membrane potential (Figure 2-8d), indicating that staurosporine-induced loss of the mitochondrial membrane potential occurred in a caspase-independent manner. This is in contrast to what was observed with DNA fragmentation (Figure 2-5c), because staurosporine directly induces the loss of the PT in a caspase-independent manner whereas DNA fragmentation requires caspase activation (58). As expected, Jurkat cells overexpressing Bcl-2 were completely resistant to the staurosporine-induced collapse of the inner membrane potential (Figure 2-8 e and f). Similarly, upon treatment with staurosporine, 87% of Jurkat cells infected with vaccinia virus maintained a TMRE-positive state (Figure 2-8 g and h), indicating that vaccinia virus infection inhibited staurosporine-induced loss of the inner mitochondrial membrane potential. The addition of cytosine arabinoside (araC), an inhibitor of virus replication and late gene expression, had no effect on the ability of vaccinia virus to inhibit staurosporine-induced loss of the inner mitochondrial membrane potential, indicating that virus replication and late gene

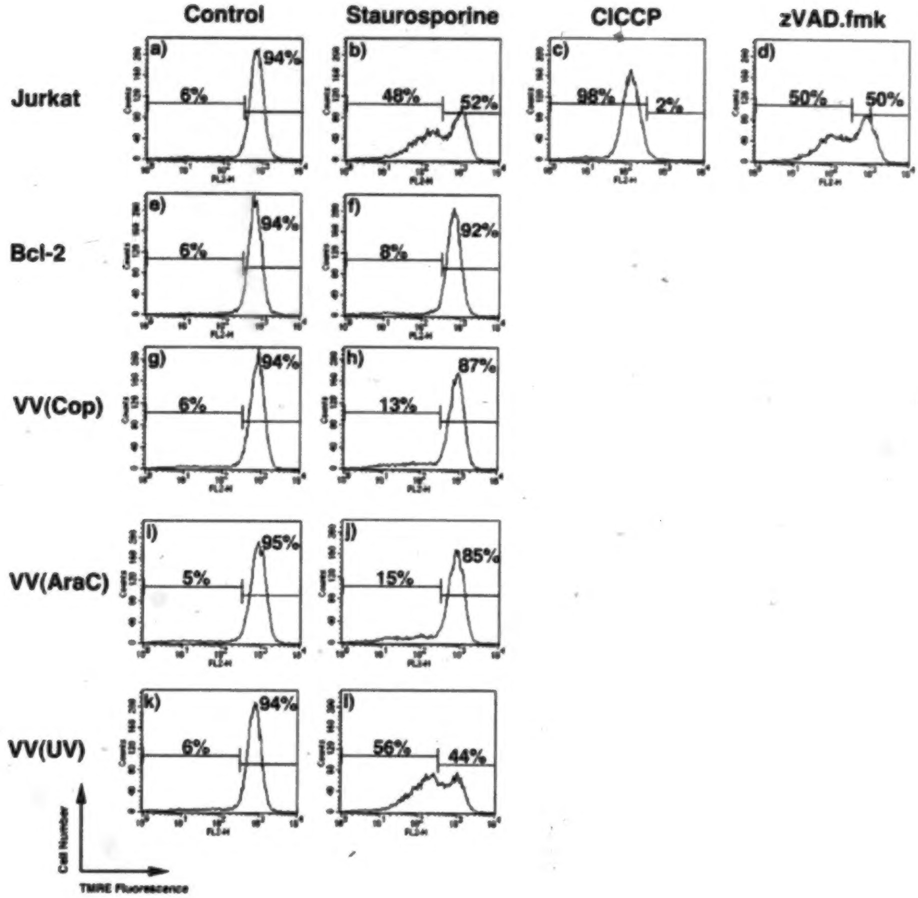


Figure 2-8 Vaccinia virus strain Copenhagen infection inhibits staurosporine-induced disruption of the mitochondrial membrane potential.
Jurkat cells were either mock infected or infected with vaccinia virus and treated with 1 μM staurosporine for 1 h, and the mitochondrial membrane potential was determined using TMRE fluorescence. (a) Untreated Jurkat cells; (b) Jurkat cells treated with staurosporine; (c) Jurkat cells treated with the membrane uncoupler ClCCP; (d) Jurkat cells treated with staurosporine in the presence of 100 μM zVAD.fmk; (e) untreated Jurkat cells overexpressing Bcl-2; (f) Jurkat cells overexpressing Bcl-2 treated with staurosporine; (g) untreated Jurkat cells infected with vaccina virus strain Copenhagen; (h) vaccinia virus-infected cells treated with staurosporine; (i) Jurkat cells infected with vaccinia virus strain Copenhagen in the presence of 40 μg of araC/ml; (j) Jurkat cells infected with vaccinia virus strain Copenhagen in the presence of araC and staurosporine; (k) untreated cells infected with UV-inactivated vaccinia virus strain Copenhagen; (l) Jurkat cells infected with UV-inactivated vaccinia virus and treated with staurosporine.

expression were not necessary (Figure 2-8 i and j). In contrast, UV inactivation of the virus resulted in reversal of this observation, clearly showing that a productive vaccinia virus infection was necessary for the inhibition (Figure 2-8 k and l).

Controlled permeabilization of the inner and outer mitochondrial membrane is known to occur as a result of opening a mitochondrial multiprotein complex known as the PTP (12, 36, 37). The PTP consists of the outer-mitochondrial-membrane-localized voltage-dependent anion carrier (VDAC), the inner-membrane-localized ANT, and the matrix protein cyclophilin D (12, 36, 37). Two accessory proteins, hexokinase and the peripheral benzodiazepine receptor, are also found associated with the PTP. Members of the Bcl-2 family associate with components of the pore and modulate pore activity, thereby inhibiting apoptosis (12, 37, 42).

Since infection of cells with vaccinia virus renders them resistant to apoptosis and inhibits disruption of the mitochondrial inner-membrane potential, we asked if vaccinia virus strain Copenhagen infection regulated apoptosis by modulating the activity of the PTP. Pore-specific ligands can act directly on components of the pore, resulting in dissipation of the inner-membrane potential and the release of cytochrome c (32, 68, 72). To ascertain if vaccinia virus infection could inhibit induction of the permeability transition and subsequent apoptosis, we treated purified mitochondria with the ANT ligand atractyloside and monitored cytochrome c release. Mitochondria were isolated from mock-infected, vaccinia virus-infected, or Bcl-2-overexpressing cells and induced to undergo permeability transition with various concentrations of atractyloside, and the translocation of cytochrome c was monitored by western blot analysis. As shown in Figure 2-9A, cytochrome c translocation from mock-infected mitochondria was detected

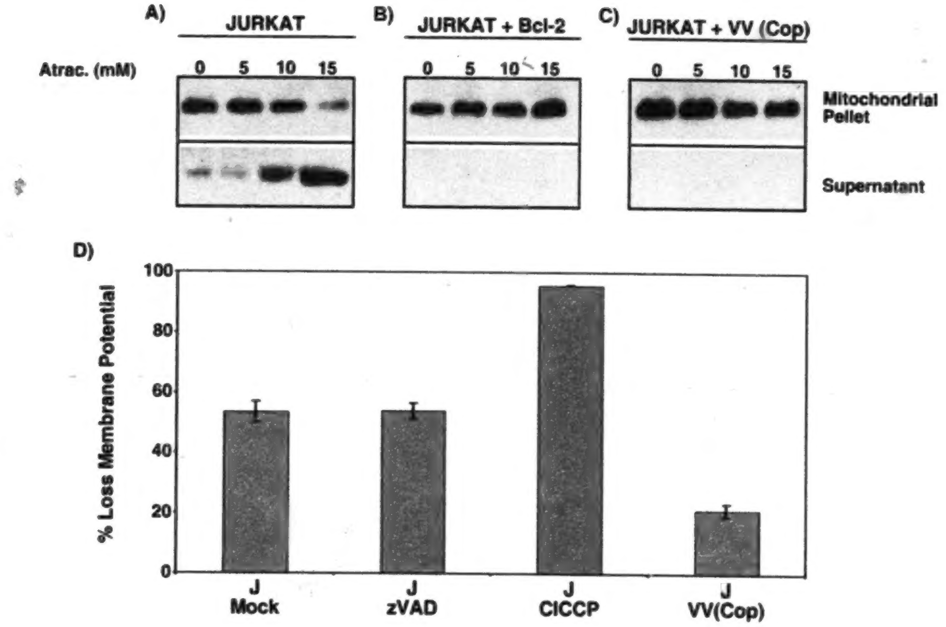


Figure 2-9 Vaccinia virus strain Copenhagen inhibits opening of the PTP.
(A through C) Atractyloside-induced cytochrome c release is inhibited by vaccinia virus strain Copenhagen infection. Mitochondria were purified and incubated at 37°C with 5, 10, or 15 mM atractyloside (Atrac.) for 40 min. Following treatment, samples were fractionated into mitochondria-containing and soluble fractions, and translocation of cytochrome c was analyzed by western blot analysis. (A) Mitochondria isolated from mock-infected Jurkat cells; (B) mitochondria isolated from mock-infected Jurkat cells overexpressing Bcl-2; (C) mitochondria isolated from vaccinia virus strain Copenhagen-infected Jurkat cells. (D) Vaccinia virus inhibits *t*-butylhydroperoxide-induced disruption of the mitochondrial membrane potential. Jurkat cells were either mock infected or infected with vaccinia virus and treated with 300 µM *t*-butylhydroperoxide for 2 h. The mitochondrial membrane potential was determined using TMRE fluorescence in the presence and absence of 100 µM zVAD.fmk. Standard deviations were generated from three independent experiments.

following treatment with 5 mM atractyloside, and increasing levels of cytochrome c release were detected with higher concentrations of atractyloside. Atractyloside-induced cytochrome c translocation was completely inhibited in mitochondria isolated from cells overexpressing Bcl-2, a known PTP-modulating protein (42) (Figure 2-9B). As shown in Figure 2-9C, atractyloside-induced cytochrome c release from mitochondria isolated from vaccinia virus-infected cells was also completely inhibited, indicating that vaccinia virus strain Copenhagen infection inhibited the onset of atractyloside-induced mitochondrial permeability transition and the subsequent translocation of cytochrome c. To further confirm that vaccinia virus infection inhibited the opening of the PTP, we treated Jurkat cells with another permeability transition inducer, t-butylhydroperoxide, and monitored mitochondrial membrane potential using TMRE fluorescence (68, 72). As shown in Figure 2-9D, treatment of mock-infected cells with *t*-butylhydroperoxide resulted in disruption of the mitochondrial membrane potential, as shown by a decrease in TMRE fluorescence. Disruption of the membrane potential following t-butylhydroperoxide treatment was not inhibited by zVAD.fmk, confirming previous findings that indicate that *t*-butylhydroperoxide does not disrupt membrane potential through caspase activation (68) (Figure 2-9D). Infection with vaccinia virus strain Copenhagen, however, prevented loss of TMRE fluorescence, indicating that vaccinia virus infection regulates the PTP (Figure 2-9D).

2.4 Discussion

In order to survive and replicate within a host, viruses possess specific strategies to circumvent the multifaceted immune response, including a variety of strategies to inhibit apoptosis (63). The detection and apoptotic elimination of virus-infected cells is mediated by specialized classes of lymphocytic cells referred to as CTL and natural killer cells. The Poxviridae, of which vaccinia virus is the prototypic member, are large double-stranded DNA viruses that encode many proteins essential for evading the antiviral immune response (56). Until now, the modulation of apoptosis by vaccinia virus has been attributed primarily to expression of the serine proteinase inhibitor Spi-2 (33, 59, 71). Previous reports, however, have suggested that vaccinia virus may foster an additional mechanism to interfere with apoptosis (15, 33, 54). In this study we report for the first time that vaccinia virus strain Copenhagen regulates the mitochondrial apoptotic pathway by inhibiting the PTP.

Since recent advances in apoptosis have [illegible]cated mitochondria as a central control point in cell death, we specifically asked [illegible]ia virus employed a mechanism to interfere with the mitochondrial component of apoptotic death (13, 37). Considering that numerous viruses encode mediators of this cascade, we predicted that vaccinia virus infection would also result in maintenance of mitochondrial integrity following the addition of a proapoptotic stimulus. To perform our studies we utilized Jurkat cells, which, due to lower levels of caspase 8 activation, undergo Fas-mediated apoptosis exclusively through the mitochondrial route (51). In addition, we chose to utilize vaccinia virus strain Copenhagen, because it is naturally devoid of the caspase 8-inhibitor CrmA/Spi-2 (19). Using this approach we found that vaccinia virus strain Copenhagen infection inhibited cell death mediated through the Fas surface receptor. Since this strain of vaccinia virus does not contain a functional Spi-2 protein, this result clearly indicated that vaccinia virus strain Copenhagen employed a novel, Spi-2-independent anti-apoptotic mechanism. Prior to this study, other researchers have suggested the existence of a Spi-2-independent anti-apoptotic effect conferred by vaccinia virus infection. Dobbelstein and Shenk found that some HeLa cells infected with a vaccinia virus lacking Spi-2 were still protected from apoptosis (15). In addition, Kettle and coworkers reported the existence of a Spi-2-independent mechanism that was capable of inhibiting cycloheximide-induced apoptosis (33). More recently, Shisler and Moss found that infection with a Spi-2-deficient virus inhibited cleavage of the caspase 3 substrate PARP (54).

To define more clearly the mechanism of vaccinia virus apoptosis inhibition, we monitored both the activation of caspase 3 and the translocation of cytochrome c in response to treatment with anti-Fas. Jurkat cells infected with vaccinia virus strain Copenhagen and treated with anti-Fas displayed reductions in both the activation of caspase 3 and the translocation of cytochrome c compared to mock-infected cells, clearly indicating that the block in apoptosis was upstream of cytochrome c release. Similarly, apoptosis induced by staurosporine was also dramatically inhibited in cells infected with vaccinia virus strain Copenhagen. Once again, both caspase 3 activation and cytochrome c release were inhibited, demonstrating that the novel mechanism employed by vaccinia virus occurred upstream of cytochrome c release. Analysis of Bid activation in an in vitro reconstitution assay indicated that vaccinia virus did not inhibit cleavage of Bid, allowing us to rule out the possiblity of interference with Bid activation. Western blot analysis of cytochrome c in the same in vitro reconstitution assay using mitochondria purified from infected and uninfected cells once again revealed that vaccinia virus interferes with the translocation of cytochrome c. Taken together, the data clearly suggest that vaccinia virus infection inhibited apoptosis by functioning directly at the mitochondria. Recently, M11L, encoded by the poxvirus myxoma virus, has been shown to localize to the mitochondria and inhibit apoptosis (17). Numerous virus-encoded Bcl-2 homologues and a novel protein from HCMV, vMIA, also inhibit apoptosis by functioning at the mitochondria (20, 64). Interestingly, no open reading frame exists in vaccinia virus strain Copenhagen that displays homology with known virus-encoded or cellular apoptotic inhibitors, suggesting that vaccinia virus has evolved a novel mechanism to inhibit cytochrome c release and apoptosis (19).

Although the exact mechanism of cytochrome c release during apoptosis is still controversial, the release of cytochrome c has been linked to disruption of the inner-mitochondrial-membrane potential (12, 36, 37, 41, 69). Dissipation of the inner-mitochondrial-membrane potential is a common and irreversible feature of apoptosis. We found that following treatment with staurosporine, cells infected with vaccinia virus strain Copenhagen retained the inner-mitochondrial-membrane potential, as monitored by TMRE fluorescence. This result supported our previous observations demonstrating that within infected cells the integrity of the mitochondria was maintained and cytochrome c translocation was inhibited. Loss of the membrane potential occurs by triggering opening of the PTP, a phenomenon known as the "permeability transition" (12, 36, 37). Since disruption of the inner-mitochondrial-membrane potential and the induction of apoptosis occurs as a result of opening of the PTP, our data suggested that perhaps vaccinia virus could maintain mitochondrial integrity by regulating the opening of the PTP. In support of this we found that vaccinia virus infection inhibited cytochrome c release from mitochondria treated with increasing amounts of atractyloside, a pore agonist known to induce the permeability transition and the release of cytochrome c (32, 68, 72). In addition, we found that vaccinia virus-infected cells treated with the pro-oxidant *t*-butylhydroperoxide, which also causes disruption of the mitochondrial transmembrane potential, demonstrated preservation of the mitochondrial inner-membrane transmembrane potential (68). Thus, vaccinia virus infection inhibited the effects mediated by both atractyloside and t-butylhydroperoxide, two reagents that trigger the permeability transition. In addition, both Bid and staurosporine can induce apoptosis via a PTP-dependent mechanism, and we found that vaccinia virus infection also inhibited apoptosis induced by these reagents (58, 67). Our data are therefore compatible with the idea that vaccinia virus has evolved a mechanism to directly modulate the permeability transition and thereby inhibit apoptosis (Figure 2-10).

Due to the multimeric nature of the PTP, multiple targets for viral modulation are possible. Both pro- and anti-apoptotic members of the Bcl-2 family associate with components of the PTP and modulate apoptosis (12, 37, 42). At least one component of the PTP has now been directly associated with virus-mediated apoptotic inhibition. The vMIA protein from HCMV associates with the ANT and inhibits apoptosis (20). Alternatively, proapoptotic viral proteins such as Vpr encoded by human immunodeficiency virus and HBx encoded by hepatitis B virus induce apoptosis by interaction with ANT and VDAC, respectively (29, 30, 47). Collectively, Vpr, HBx, and vMIA modulate apoptosis by interacting with components of the PTP and regulating the pore complex. Thus, a similar scenario to account for vaccinia virus interference with the mitochondrial apoptotic pathway is likely, and we are currently looking for vaccinia virus proteins that interact with known components of the pore. At present, however, the exact

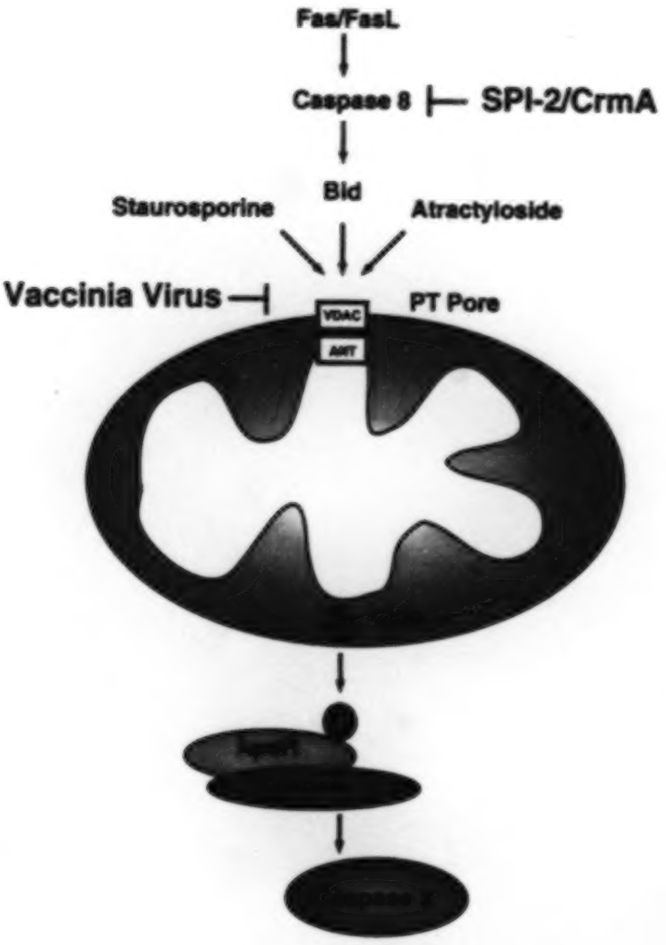


Figure 2-10 Model of vaccinia virus-mediated apoptosis inhibition.
Fas initiated apoptosis occurs via activation of caspase 8 and by the subsequent proteolytic cleavage of Bid. Once cleaved, Bid translocates to the mitochondria, resulting in a loss of the inner-mitochondrial-membrane permeability transition and the release of cytochrome c. The release of cytochrome c (Cyto. c) results in activation of caspase 9 via interaction with the adapter molecule Apafl and the subsequent activation of caspase 3. Additionally, loss of the inner-membrane permeability transition can be triggered by staurosoporine and atractyloside. Vaccinia virus infection manipulates this pathway at two separate points. First, the vaccinia virus-encoded serine protease inhibitor CrmA/SPI-2 inhibits the activity of caspase 8. Second, vaccinia virus infection also inhibits apoptosis by modulating the PTP (PT pore), thereby preventing the loss of cytochrome c and activation of caspase 9.

composition of the PTP is still controversial, and the identification of a novel vaccinia virus-encoded protein and its exact mechanism of action will lead to a clearer understanding of the PTP and its regulation in the future. In addition, further investigation into vaccinia virus modulation of the PTP and apoptosis will result in valuable information regarding virus-host interactions and the mechanisms of cell death.

2.5 References

1. **Afonso, C. L., J. G. Neilan, G. F. Kutish, and D. L. Rock.** 1996. An African swine fever virus Bcl-2 homolog, 5-HL, suppresses apoptotic cell death. J Virol **70:**4858-63.
2. **Barry, M., and G. McFadden.** 1998. Apoptosis regulators from DNA viruses. Curr Opin Immunol **10:**422-30.
3. **Barry, M., J. A. Heibein, M. J. Pinkoski, S. F. Lee, R. W. Moyer, D. R. Green, and R. C. Bleackley.** 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol **20:**3781-94.
4. **Barry, M., J. Heibein, M. Pinkoski, and R. C. Bleackley.** 2000. Quantitative measurement of apoptosis induced by cytotoxic T lymphocytes. Methods Enzymol **322:**40-46.
5. **Bertin, J., R. C. Armstrong, S. Ottilie, D. A. Martin, Y. Wang, S. Banks, G. H. Wang, T. G. Senkevich, E. M. Alnemri, B. Moss, M. J. Lenardo, K. J. Tomaselli, and J. I. Cohen.** 1997. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. Proc Natl Acad Sci USA **94:**1772-76.
6. **Bertrand, R., E. Solary, P. O'Connor, K. W. Kohn, and Y. Pommier.** 1994. Induction of a common pathway of apoptosis by staurosporine. Exp Cell Res **211:**314-21.
7. **Brun, A., C. Rivas, M. Esteban, J. M. Escribano, and C. Alonso.** 1996. African swine fever virus gene A179, a viral homologue of bcl-2, protects cells from programmed cell death. Virology **225:**227-30.
8. **Bump, N. J., M. Hackett, M. Hugunin, S. Seshagiri, K. Brady, P. Chen, C. Ferenz, S. Franklin, T. Ghayur, P. Li, P. Licari, J. Mankovich, L. Shi, A. H. Greenberg, L. K. Miller, and W. W. Wong.** 1995. Inhibition of ICE family proteases by baculovirus anti-apoptotic protein p35. Science **269:**1885-88.
9. **Caputo, A., J. C. Parrish, M. N. G. James, J. C. Powers, and R. C. Bleackley.** 1999. Electrostatic reversal of serine proteinase substrate specificity. Proteins **35:**415-24.
10. **Chao, D. T., and S. J. Korsmeyer.** 1998. BCL-2 family: regulators of cell death. Annu Rev Immunol **16:**395-19.
11. **Clem, R. J., and C. S. Duckett.** 1997. The *iap* genes: unique arbitrators of cell death. Trends Cell Biol **7:**337-39.
12. **Crompton, M.** 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem J **341:**233-49.
13. **Desagher, S., and J. C. Martinou.** 2000. Mitochondria as the central control point of apoptosis. Trends Cell Biol **10:**369-77.
14. **Deveraux, Q. L., and J. C. Reed.** 1999. IAP family proteinssuppressors of apoptosis. Genes Dev **13:**239-52.
15. **Dobbelstein, M., and T. Shenk.** 1996. Protection against apoptosis by the vaccinia virus SPI-2 (B13R) gene product. J Virol **70:**6479-85.
16. **Ehrenberg, B., V. Montana, M. D. Wei, J. P. Wuskell, and L. M. Loew.** 1988. Membrane potential can be determined in individual cells from the Nernstian distribution of cationic dyes. Biophys J **53:**785-94.
17. **Everett, H., M. Barry, S. F. Lee, X. Sun, K. Graham, J. Stone, R. C. Bleackley, and G. McFadden.** 2000. M11L: a novel mitochondria-localized protein of myxoma virus that blocks apoptosis of infected leukocytes. J Exp Med **191:**1487-98.
18. **Farkas, D. L., M. D. Wei, P. Febbroriello, J. H. Carson, and L. M. Loew.** 1998. Simultaneous imaging of cell and mitochondrial membrane potentials. Biophys J **56:**1053-69.
19. **Goebel, S. J., G. P. Johnson, M. E. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete DNA sequence of vaccinia virus. Virology **179:**247-66.
20. **Goldmacher, V. S., L. M. Bartle, A. Skaletskaya, C. A. Dionne, N. L. Kedersha, C. A. Vater, J. Han, R. J. W. Lutz, S. Watanabe, E. D. Cahir McFarland, E. D. Kieff, E. S. Mocarski, and T. Chittenden.** 1999. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc Natl Acad Sci USA **96:**12536-41.

21. **Gross, A., J. M. McDonnell, and S. J. Korsmeyer.** 1999. Bcl-2 family members and the mitochondria in apoptosis. Genes Dev **13:**1899-11.

22. **Gross, A., X. M. Yin, K. Wang, M. C. Wei, J. Jockel, C. Milliman, H. Erdjument-Bromage, P. Tempst, and S. J. Korsmeyer.** 1999. Caspase cleaved Bid targets mitochondria and is required for cytochrome *c* release, while Bcl-Xl prevents this release but not tumor necrosis factor-R1/Fas Death. J Biol Chem **274:**1156-63.

23. **Hanna, W. L., X. Zhang, J. Turbov, U. Winkler, D. Hudig, and C. J. Froelich.** 1993. Rapid purification of cationic granule proteases: application to human granzymes. Protein Expr Purif **4:**398-04.

24. **Heibein, J. A., I. S. Goping, M. Barry, M. J. Pinkoski, G. C. Shore, D. R. Green, and R. C. Bleackley.** 2000. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members Bid and Bax. J Exp Med **192:**1391-01.

25. **Heibein, J. A., M. Barry, B. Motyka, and R. C. Bleackley.** 1999. Granzyme B-induced loss of mitochondrial inner membrane potential (m) and cytochrome c release are caspase-independent. J Immunol **163:**4683-93.

26. **Hnatiuk, S., M. Barry, W. Zeng, L. Liu, A. Lucas, D. Percy, and G. McFadden.** 1999. Role of the C-terminal RDEL motif of the myxoma virus M-T4 protein in terms of apoptosis regulation and viral pathogenesis. Virology **263:**290-06.

27. **Hu, F. Q., C. A. Smith, and D. J. Pickup.** 1994. Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. Virology **204:**343-56.

28. **Hu, S., C. Vincenz, M. Buller, and V. M. Dixit.** 1997. A novel family of viral death effector domain-containing molecules that inhibit both CD95- and tumor necrosis factor receptor-1-induced apoptosis. J Biol Chem **272:**9621-24.

29. **Jacotot, E., K. F. Ferri, C. El Hamel, C. Brenner, S. Druillennec, J. Hoebeke, P. Rustin, D. Metivier, C. Lenoir, M. Geuskens, H. L. Vieira, M. Loeffler, A. S. Belzacq, J. P. Briand, N. Zamzami, L. Edelman, Z. H. Xie, J. C. Reed, B. P. Roques, and G. Kroemer.** 2001. Control of mitochondrial membrane permeabilization by adenine nucleotide translocator interacting with HIV-1 viral protein vpr and Bcl-2. J Exp Med **193:**509-19.

30. **Jacotot, E., L. Ravagnan, M. Loeffler, K. F. Ferri, H. L. A. Vieira, N. Zamzami, P. Costantini, S. Druillennec, J. Hoebeke, J. P. Briand, T. Irinopoulou, E. Daugas, S. A. Susin, D. Cointe, Z. H. Xie, J. C. Reed, B. P. Roques, and G. Kroemer.** 2000. The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. J Exp Med **191:**33-45.

31. **Kägi, D., B. Ledermann, K. Burki, R. M. Zinkernagel, and H. Hengartner.** 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. Annu Rev Immunol **14:**207-32.

32. **Kantrow, S. P., and C. A. Piantadosi.** 1997. Release of cytochrome c from liver mitochondria during permeability transition. Biochem Biophys Res Commun **232:**669-71.

33. **Kettle, S., A. Alcami, A. Khanna, R. Ehret, C. Jassoy, and G. L. Smith.** 1997. Vaccinia virus serpin B13R (Spi-2) inhibits interleukin-1 converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1-induced fever. J Gen Virol **78:**677-85.

34. **Kettle, S., N. W. Blake, K. M. Law, and G. L. Smith.** 1995. Vaccinia virus serpins B13R (SPI-2) and B22R (SPI-1) encode Mr 38.5 and 40K, intracellular polypeptides that do not affect virus virulence in a murine intranasal model. Virology **206:**136-47.

35. **Kluck, R. M., E. Bossy-Wetzel, D. R. Green, and D. D. Newmeyer.** 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science **275:**1132-36.

36. **Kroemer, G., B. Dallaporta, and M. Resche-Rigon.** 1998. The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol **60:**619-42.

37. **Kroemer, G., and J. C. Reed.** 2000. Mitochondrial control of cell death. Nat. Med. **6:**513-519.

38. **Li, H., H. Zhu, C. Xu, and J. Yuan.** 1998. Cleavage of Bid by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell **94:**491-01.

39. **Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang.** 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell **91:**479-89.

40. **Luo, X., I. Budihardjo, H. Zou, C. Slaughter, and X. Wang.** 1998. Bid, a Bcl-2 interacting protein, mediates cytochrome c release from the mitochondria in response to activation of cell surface death receptors. Cell **94:**481-90.

41. **Marchetti, P., T. Hirsch, N. Zamzami, M. Castedo, D. Decaudin, S. A. Susin, B. Masse, and G. Kroemer.** 1996. Mitochondrial permeability transition triggers lymphocyte apoptosis. J Immunol **157:**4830-36.

42. **Marzo, I., C. Brenner, N. Zamzami, S. A. Susin, G. Beutner, D. Brdiczka, R. Remy, Z.-H. Xie, J. C. Reed, and G. Kroemer.** 1998. The permeability transition pore complex: a target for apoptosis regulation by caspases and Bcl-2 related proteins. J Exp Med **187:**1261-71.

43. **Metivier, D., B. Dallaporta, N. Zamzami, N. Larochette, S. A. Susin, I. Marzo, and G. Kroemer.** 1998. Cytofluorometric detection of mitochondrial alterations in early CD95/Fas/APO-1-triggered apoptosis of Jurkat T lymphoma cells. Comparison of seven mitochondrion-specific fluorochromes. Immunol Lett **61:**157-63.

44. **Murphy, K. M., U. N. Streips, and R. B. Lock.** 2000. Bcl-2 inhibits a Fas-induced conformational change in the Bax N terminus and Bax mitochondrial translocation. J Biol Chem **275:**17225-28.

45. **Nicholson, D. W., and N. A. Thornberry.** 1997. Caspases: killer proteases. Trends Biochem Sci **22:**299-06.

46. **Nogal, M. L., G. Gonzalez de Buitrago, C. Rodriguez, B. Cubelos, A. L. Carrascosa, M. L. Salas, and Y. Revilla.** 2001. African swine fever virus IAP homologue inhibits caspase activation and promotes cell survival in mammalian cells. J Virol **75:**2535-43.

47. **Rahmani, Z., K. W. Huh, R. Lasher, and A. Siddiqui.** 2000. Hepatitis B virus X protein colocalizes to mitochondria with a human voltage-dependent anion channel, HVDAC3, and alters the transmembrane potential. J Virol **74:**2840-46.

48. **Roulston, A., R. C. Marcellus, and P. E. Branton.** 1999. Viruses and apoptosis. Annu Rev Microbiol **53:**577-28.

49. **Samali, A., J. Cai, B. Zhivotovsky, D. P. Jones, and S. Orrenius.** 1999. Presence of a pre-apoptotic complex of pro-caspase 3, Hsp60 and Hsp10 in the mitochondrial fraction of Jurkat cells. EMBO J **18:**2040-48.

50. **Scaduto, R. C., Jr., and L. W. Grotyohann.** 1999. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J **76:**469-77.

51. **Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K. J. Tomaselli, K. M. Debatin, P. H. Krammer, and M. E. Peter.** 1998. Two CD95 (APO-1/Fas) signaling pathways. EMBO J **17:**1675-87.

52. **Schreiber, M., K. Rajarathnam, and G. McFadden.** 1996. Myxoma virus T2 protein, a tumor necrosis factor (TNF) receptor homolog, is secreted as a monomer and dimer that each bind rabbit TNF, but the dimer is a more potent TNF inhibitor. J Biol Chem **271:**13333-41.

53. **Shisler, J., C. Yang, B. Walter, C. F. Ware, and L. R. Gooding.** 1997. The adenovirus E3-10.4K/14.5K complex mediates loss of cell surface Fas (CD95) and resistance to Fas-induced apoptosis. J Virol **71:**8299-06.

54. **Shisler, J. L., and B. Moss.** 2001. Molluscum contagiosum virus inhibitors of apoptosis: the MC159 v-FLIP protein blocks Fas-induced activation of procaspases and degradation of the related MC160 protein. Virology **282:**14-25.

55. **Smith, C. A., T. Davis, J. M. Wignall, W. S. Din, T. Farrah, C. Upton, G. McFadden, and R. G. Goodwin.** 1991. T2 open reading frame from the Shope fibroma virus encodes a soluble form of the TNF receptor. Biochem Biophys Res Commun **176:**335-42.

56. **Smith, G. L., J. A. Symons, A. Khanna, A. Vanderplasschen, and A. Alcami.** 1997. Vaccinia virus immune evasion. Immunol Rev **159:**137-54.

57. **Stuart, D., K. Graham, M. Schreiber, C. Macaulay, and G. McFadden.** 1991. The target DNA sequence for resolution of provirus replicative intermediates is an active late promoter. J Virol **5:**61-70.

58. **Tafani, M., D. A. Minchenko, A. Serroni, and J. L. Farber.** 2001. Induction of the mitochondrial permeability transition mediates the killing of HeLa cells by staurosporine. Cancer Res **61:**2459-66.

59. **Tewari, M., and V. M. Dixit.** 1995. Fas- and tumor necrosis factor-induced apoptosis inhibited by the poxvirus *crmA* gene product. J Biol Chem **270:**3255-60.

60. **Thome, M., P. Schneider, K. Hofmann, H. Fickenscher, E. Meinl, F. Neipel, C. Mattmann, K. Burns, J. L. Bodmer, M. Schroter, C. Scaffidi, P. H. Krammer, M. E. Peter, and J. Tschopp.** 1997. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature **386:**517-21.

61. **Thornberry, N. A., and Y. Lazebnik.** 1998. Caspases: enemies within. Science **281:**1312-16.

62. **Tollefson, A. E., T. W. Hermiston, D. L. Lichtenstein, C. F. Colle, R. A. Tripp, T. Dimitrov, K. Toth, C. E. Wells, P. C. Doherty, and W. M. Wold.** 1998.Forced degradation of Fas inhibits apoptosis in adenovirus-infected cells. Nature **392:**726-30.

63. **Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh.** 2000. Viral subversion of the immune system. Annu Rev Immunol **18:**861-26.

64. **Tschopp, J., M. Thome, K. Hofmann, and E. Meinl.** 1998. The fight of viruses against apoptosis. Curr Opin Genet Dev **8:**82-87.

65. **White, E., P. Sabbatini, M. Debbas, W. S. Wold, D. I. Kusher, and L. R. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. Mol Cell Biol **12:**2570-80.

66. **Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang.** 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science **275:**1129-32.

67. **Zamzami, N., C. El Hamel, C. Maisse, C. Brenner, C. Munoz-Pinedo, A. S. Belzacq, P. Costantini, H. Vieira, M. Loeffler, G. Molle, and G. Kroemer.** 2000. Bid acts on the permeability transition pore complex to induce apoptosis. Oncogene **19:**6342-50.

68. **Zamzami, N., I. Marzo, S. A. Susin, C. Brenner, N. Larochette, P. Marchetti, J. Reed, R. Kofler, and G. Kroemer.** 1998. The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mtiochondrial permeability transition. Oncogene **16:**1055-63.

69. **Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J. L. Vayssiere, P. X. Petit, and G. Kroemer.** 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med **181:**1661-72.

70. **Zhou, Q., J. F. Krebs, S. J. Snipas, A. Price, E. S. Alnemri, K. J. Tomaselli, and G. S. Salvesen.** 1998. Interaction of the baculovirus anti-apoptotic protein p35 with caspases. Specificity, kinetics, and characterization of the caspase/p35 complex. Biochemistry **37:**10757-65.

71. **Zhou, Q., S. Snipas, K. Orth, M. Muzio, V. M. Dixit, and G. S. Salvesen.** 1997. Target protease specificity of the viral serpin CrmA: analysis of five caspases. J Biol Chem **272:**7797-00.

72. **Zoratti, M., and I. Szabo.** 1995. The mitochondrial permeability transition. Biochim Biophys Acta **1241:**139-76.

Chapter 3: Vaccinia Virus Encodes a Novel Mitochondrial Associated Inhibitor of Apoptosis

Preface

The data presented in this chapter was originally submitted to EMBO Journal. A modified version of this chapter has been published; Wasilenko ST, Stewart TL, Meyers AF, Barry M. PNAS, 2003, 100: 14345-50. The data presented in all figures was generated by myself with the exception of Figure 3-5A and Figure 3-7 which were carried out by Tara Stewart. I wrote the first draft of the manuscript. A major editorial contribution from my supervisor, Dr. Michele Barry, led to the final version of the paper.

3.1 Introduction

Apoptosis or programmed cell death is a major antiviral response utilized by multicellular organisms for the coordinated removal of infected cells (4, 26). Apoptosis involves the activation of a family of cysteine proteases, referred to as caspases, that mediate the ordered dismantling of infected cells resulting in morphological changes such as chromatin condensation, DNA fragmentation and plasma membrane blebbing. Recent advances have also identified mitochondria as important regulating organelles during apoptosis (14, 33, 41, 64). Apoptosis results in mitochondrial alterations, such as loss of the mitochondrial membrane potential ($\Delta\Psi m$), production of reactive oxygen species and release of pro-apoptotic proteins (33, 64). Pro-apoptotic proteins released from the mitochondria include SMAC, HtrA2, apoptosis inducing factor (AIF), endonuclease G and cytochrome c (64). Once released, SMAC and HtrA2 effectively inhibit cellular inhibitors of apoptosis (IAP) and subsequent caspase regulation, while AIF and endonuclease G are important for DNA fragmentation (15, 35, 37, 54, 55). Mitochondrial-released cytochrome c initiates caspase activation through an interaction with the adapter molecule Apaf-1 subsequently promoting recruitment and activation of caspase 9 in an ATP-dependent manner (36).

Both pro- and anti-apoptotic members of the Bcl-2 family tightly regulate the mitochondrial checkpoint (10, 11, 24, 41). Anti-apoptotic family members such as Bcl-2 and Bcl-xL localize to the outer membrane of mitochondria and inhibit the release of pro-apoptotic proteins such as cytochrome c. Following an apoptotic stimulus, pro-apoptotic members of the Bcl-2 family, such as Bid, Bax and Bad, translocate from the cytoplasm to the mitochondrion to induce the release of pro-apoptotic proteins (25, 34, 38, 69).

Although members of the Bcl-2 family are known to be critical regulators of the mitochondrial checkpoint, their functional mechanism remains elusive and controversial.

Successful viral infection requires that viruses tolerate multiple assaults inflicted by the host immune response. To combat the multifaceted host immune response many viruses have evolved strategies to modulate and inhibit immune defense mechanisms (58). Poxviruses represent a large family of viruses that infect both vertebrates and invertebrates and unlike other DNA viruses, replicate within the cytoplasm of infected cells independent of host cell factors. Vaccinia virus is the prototypic member of the family and is the most widely studied, however, the most notorious member of the poxvirus family is variola virus, the causative agent of smallpox (52). Poxviruses have proven to be masters at immune evasion by encoding a wealth of proteins that regulate cytokine pathways, antigen presentation, and apoptosis (19, 30, 50).

To efficiently replicate and disseminate within the host, virus manipulation of the apoptotic response is essential. Multiple viruses recognize the importance of controlling apoptosis and have evolved mechanisms that target crucial components within the apoptotic cascade (7, 19, 47, 59). One of the best-studied virus-encoded anti-apoptotic proteins is the cowpox virus-encoded inhibitor cytokine response modifier A (CrmA) which effectively inhibits caspases 1 and 8 (32, 57, 70). In addition to direct caspase inactivation, viruses also regulate the mitochondrial checkpoint by encoding obvious Bcl-2 homologous proteins that function at the mitochondria to inhibit apoptosis. For example, Bcl-2 homologues exist in fowlpox virus, adenovirus, African swine fever virus and multiple members of the gammaherpes virus family (13). More recently, novel viral proteins that localize to the mitochondria and inhibit apoptosis but lack significant homology to Bcl-2 have been identified. Kaposi's sarcoma associated herpesvirus (KSHV) encodes K7, a homologue of human survivin that localizes to intracellular membranes, including the mitochondria (20, 63). The UL37 gene product encoded by human cytomegalovirus (HCMV), referred to as vMIA for mitochondrial-localized inhibitor of apoptosis, lacks homology to Bcl-2 but localizes to the mitochondria and inhibits the mitochondrial release of cytochrome c (22, 61). A third protein, M11L, which is encoded by the rabbit specific poxvirus, myxoma virus, also lacks homology to members of the Bcl-2 family but localizes to the mitochondrion via a C-terminal mitochondrial targeting sequence where it effectively inhibits apoptosis (17, 18). The identification of these novel virus-encoded anti-apoptotic proteins clearly suggests the presence of other previously unidentified inhibitors of the mitochondrial apoptotic checkpoint.

The importance of mitochondria during apoptosis, as well as the lack of any homologous mitochondrial-localized anti-apoptotic genes in the genome of vaccinia virus, led us to speculate that vaccinia virus would encode a novel protein to inhibit the mitochondrial component of apoptosis. In support of this, we previously demonstrated that Jurkat cells infected with vaccinia virus strain Copenhagen, which is devoid of the functional caspase inhibitor, CrmA, were protected from apoptosis induced by staurosporine and anti-Fas (65). Furthermore, cells infected with vaccinia virus strain Copenhagen were completely resistant to loss of mitochondrial inner membrane potential and release of cytochrome c (65). In this study we report the identification and characterization of a novel vaccinia virus-encoded protein, F1L, that localizes exclusively to mitochondria where it functions to regulate the apoptotic cascade.

3.2 Materials and Methods

Cells and Viruses.

Jurkat cells were grown in RPMI 1640 medium (Gibco Invitrogen Inc.) supplemented with 10% fetal bovine serum (FBS) (Gibco Invitrogen Inc.), 100μM 2-mercaptoethanol, 50U/mL penicillin, and 50μg/mL streptomycin (RHFM). Jurkat cells stably expressing Bcl-2 were generated as previously described and maintained in RHFM (5). Bak and Bax deficient Jurkat cells, a gift from Dr Hanna Rabinowich, The University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, were maintained in RHFM (62). HeLa and Cos-1 cells were grown in DMEM (Gibco Invitrogen Inc.) supplemented with 10% FBS (Gibco Invitrogen Inc.), 2mM L-glutamine, 50U/mL penicillin, and 50μg/mL streptomycin. Baby green monkey cells (BGMK) were routinely grown in DMEM supplemented with 10% newborn calf serum (Gibco Invitrogen Inc.), 2mM L-glutamine, 50U/mL penicillin, and 50μg/mL streptomycin.

Recombinant vaccinia virus strain Copenhagen expressing β-galactosidase, VV65, was provided by Dr. G. McFadden, Robarts Research Institute, London, Ontario, Canada (27). The deletion viruses VV811 and VV759 were a generous gift from Dr. Enzo Paoletti (45). All viruses were routinely propagated in BGMK cells and purified as previously described (53). VV811 was additionally purified using a continuous sucrose gradient (29).

Virus Infection.

Jurkat or HeLa cells were infected with VV65, VV811, or VV759 at a multiplicity of infection (MOI) of either 1 or 10 plaque forming units (pfu) per cell in 200μL RHFM media at 37°C. After one hour, the cells were supplemented with either complete RHFM media or complete DMEM for 4 hours and incubated at 37°C and 5% CO_2 prior to the induction of apoptosis.

Plasmid Construction.

DNA was purified from VV65-infected BGMK cells as previously described (6). The F1L open reading frame was amplified from the virus DNA by polymerase chain reaction (PCR) using the forward oligonucleotide (5'-CTCGAGATGTTGTCGATGTTTATG) containing a XhoI restriction site and the reverse oligonucleotide (5'-GGATCCTTATCCTATCATGTATTT) containing a BamHI restriction site. The amplified F1L product was ligated into the TA cloning vector pGEMT (Promega Corporation) to construct pGEMTF1L. F1L was subsequently subcloned into the XhoI and BamHI sites of pEGFP-C3 (Clontech Laboratories Inc.) to construct pEGFP-F1L. A truncated version of F1L(1-206) was generated by PCR using the forward oligonucleotide (5'-CTCGAGATGTTGTCGATGTTTATG) containing a XhoI restriction site and the reverse oligonucleotide (5'GGATCCTTACTTTAG ATATTCACGCGTGCT) containing a BamHI restriction site. The amplified product, F1L(1-206), was ligated into pGEMT and subcloned into the XhoI and BamHI sites of pEGFP-C3(Clontech Laboratories Inc.) to create pEGFP-F1L(1-206). A N-terminal deleted version of F1L(199-226) was generated by PCR using the forward primer (5'CTCGAGACTAGCACGCGTGAATAT) containing a XhoI restriction site and the reverse oligonucleotide (5'GGATCCTTATCCTATCATGTATTT) containing a BamHI restriction site. The amplified product, F1L(199-226) was ligated into pGEMT and subcloned into the XhoI and BamHI sites of pEGFP-C3 (Clontech Laboratories Inc.) to create pEGFP-F1L(199-226). The plasmid EGFP-Bcl2 was a generous gift from Dr. C. Bleackley, University of Alberta, Edmonton, Alberta, Canada. For the infection/transfection assays, the plasmid pSC66 was used to express EGFP and F1L under the control of the early/late p7.5 poxviral promoter. To create pSC66, the EGFP coding sequence was liberated from pEGFP-N3 (Clontech Laboratories Inc.) with SalI and NotI and subsequently subcloned into pSC66 to create pSC66-EGFP. To create pSC66-F1L, the F1L open reading frame was amplified by PCR from pEGFP-F1L with the forward oligonucleotide (5’-GCGGCCGCATGTTGTCGATGTTTATG) containing a NotI restriction site and the reverse oligonucleotide (5’-GGATCCTTATCCTATCATGTATTT) containing a BamHI site. The amplified F1L product was subcloned into the TA cloning vector pGEMT vector (Promega Corporation). To create pSC66-F1L, the F1L coding sequence was liberated from pGEMT-F1L and subcloned into the NotI site of pSC66. For construction of pSC66-F1L-Flag-N, which contains a Flag-tag at the N-terminus of F1L, the F1L open reading frame was amplified by PCR using pEGFP-F1L as the template with the forward oligonucleotide F1L-Flag-N-SalI (5’-GTCGACATGGACTACAAAGACGATGACGACAAGTTGTCGATGTTTATGTGT-3’) containing a SalI restriction site and a Flag epitope tag and the reverse oligonucleotide (5’-CTCGAGTTATCCTATCATGTATTT-3’) containing a XhoI restriction site. The amplified product was inserted into the pGEMT cloning vector (Promega Corporation) and subcloned into the SalI and NotI sites of pSC66 to generate pSC66-F1L-Flag-N. For stable expression, a copy of the F1L open reading frame containing a 5’ influenza HA tag was engineered by PCR from pEGFP-F1L using the forward oligonucleotide (5’-

GTCGACATGTACCCATACGATGTTCCAGATTACGCTCTTATGTTGTCGATGTTTATG-3') containing a SalI site and the reverse oligonucleotide (5'-CTCGAGTTATCCTATCATGTATTT-3') containing a XhoI restriction site. The amplified product was subcloned into the TA cloning vector pGEMT (Promega Corporation). The HA-tagged F1L open reading frame was subsequently subcloned into the SalI site of the eukaryotic expression vector BMGneo and designated BMGNeo-F1L-HA-N (31).

Generation of Stable Transfected Cell Lines.

Jurkat cells (5×10^6) were stably transfected with 20μg of BMGneo or BMGneo-F1L-HA-N by electroporation at 250V with 3 × 8 ms pulses (BTX Division of Genetronics). Stable transfectants were selected in 1mg/mL G418 (Gibco Invitrogen Corporation) and subsequently cloned by serial dilution. Stable clones were maintained in RHFM supplemented with 800μg/mL G418.

Apoptosis Induction.

Jurkat cells were induced to undergo apoptosis by the addition of 125ng/mL or 250ng/mL anti-Fas (clone CH11) (Upstate Biotechnology), 1 to 2.5μM of staurosporine (Sigma Chemical Co.) or by infection with VV811 at an MOI of 10 pfu per cell for 15 hours. HeLa cells were treated with 125ng/mL anti-Fas (clone CH11) (Upstate Biotechnology) and 5μg/mL cycloheximide (Sigma Chemical Co.). To inhibit caspase activity, 100μM zVAD.fmk (Kamiya Biomedical) was added 30 minutes prior to the addition of apoptotic stimuli.

Detection of DNA Fragmentation.

DNA fragmentation was assessed using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method (Roche Diagnostics Co.). Briefly, cells were harvested, washed in PBS containing 1% FBS, fixed in 2% paraformaldehyde, and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. Fixed and permeabilized cells were incubated for 1 hour at 37°C in a solution containing 25mM Tris (pH 6.6), 200mM cacodylate, 1mM $CoCl_2$, 0.6nM fluorescein-12-dUTP, and 25U of terminal deoxynucleotidyltransferase (Roche Diagnostics Co.). Analysis was performed on a Becton Dickinson FACScan equipped with an argon laser at 15mV with an excitation wavelength of 488nm. Emission wavelengths were detected through the FL-1 channel equipped with a 530nm filter (band pass, 20nm). Data was acquired on a minimum of 10,000 cells per sample with light scatter signals at linear gain and fluorescence signals at logarithmic gain.

Cellular Fractionation.

Cellular fractionation into cytosolic and membranous fractions was performed as previously described (65). Following the addition of a pro-apoptotic stimulus, 2×10^6 cells were permeabilized by incubation in digitonin lysis buffer containing 75mM NaCl, 1mM NaH_2PO_4, 8mM Na_2HPO_4, 250mM sucrose, and 190μg/mL digitonin (Sigma Chemical Co.). Cells were incubated on ice for 10 minutes after which the mitochondria-containing pellet and the cytosolic supernatant were separated by centrifugation at 10,000×g for 5 minutes. Mitochondrial pellets were resuspended in 0.1% Triton X-100, and 25mM Tris, pH 8.0, prior to SDS PAGE analysis. Cytochrome c release was monitored by immunoblotting both the supernatant and membranous fractions. The supernatant fraction was also used to assess caspase activation by immunoblot analysis.

Infection and Transfection Assay.

Transient transfection of Jurkat cells or Jurkat cells overexpressing Bcl-2 with either pSC66-EGFP and pSC66-F1L or pSC66-EGFP and pSC66 in the presence of VV811 was performed as previously described (23). Cells (1×10^6) were transfected with 5µg DNA using DMRIE-C (Invitrogen Life Technologies) according to the manufacturers protocol. Concurrent with the transfection process, Jurkat cells were infected with VV811 at a MOI of 1 pfu/cell in 600µL OptiMem media (Invitrogen Life Technologies). Four hours post infection, cells were removed from OptiMEM medium and supplemented with complete RHFM for an additional 2 to 4 hours before the addition of staurosporine or anti-Fas.

Transient Transfections.

HeLa cells were seeded in 6 well plates at a density of 5×10^5. At 70% confluency, the cells were transfected with 2µg of DNA using Lipofectamine 2000 and OptiMEM (GIBCO BRL Technologies Inc.) according to the manufacture's protocol. Following a 20 hour incubation, cells were induced to undergo apoptosis with anti-Fas and cycloheximide.

Measurement of Mitochondrial Membrane Potential.

Changes in mitochondrial membrane potential were quantified in transfected and infected cells by staining cells with tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) (16, 43). Cells were loaded with TMRE by incubating cells in RHFM media containing 0.2μM TMRE for 30 minutes at 37°C in 5% CO_2. As a control, cells were also treated with a membrane uncoupler, carbonyl cyanide m-chlorophenyl hydrazone (ClCCP) (Sigma Chemical Co.) at a concentration of 50μM for 15 minutes at 37°C in 5% CO_2. To trigger apoptosis, cells were treated with either 1μM staurosporine for 2 hours, 250ng/ml anti-Fas for 8 hours, or 125ng/mL anti-Fas and 5μg/mL cycloheximide for 8 hours. Prior to flow cytometric analysis, cells were washed with PBS containing 1% FBS. TMRE fluorescence was acquired through the FL-2 channel equipped with a 585 filter (42nm band pass). Data was acquired with fluorescence signals at logarithmic gain. When required, detection of EGFP positive cells was performed on a Becton Dickinson FACScan equipped with an argon laser with 15mV of excitation at 488nm. Emission wavelengths were detected through the FL-1 channel equipped with a 530nm filter (20nm band pass). Data was analyzed with CellQuest software. the loss of inner mitochondrial membrane potential in positively transfected cells was calculated for the EGFP positive population using the following equation: (number EGFP+TMRE-cells/total of number EGFP+ cells) × 100.

Confocal Microscopy.

Live cell imaging and localization studies of F1L were performed by seeding either Cos-1 or HeLa cells on 1mm glass coverslips. At approximately 60% confluency cells were transfected with 2μg of pEGFP-C3, pEGFP-F1L, pEGFP-F1L(1-206), or pEGFP-F1L(199-206) using Lipofectamine 2000 following the manufacturer's protocol (Invitrogen Life Technologies). Sixteen hours after transfection cells were either treated with 15ng/mL Mitotracker Red CMXRos (Molecular Probes) for 30 minutes at 37°C in 5% CO_2 to label mitochondria. Alternatively, cells were treated with 125ng/mL anti-Fas (clone CH11) and 5μg/mL cycloheximide (Sigma Chemical Co.) for 3 hours to induce apoptosis.

To study the localization of F1L in the presence of vaccinia virus infection, 1×10^6 HeLa cells were seeded on 1mm glass coverslips. HeLa cells were transiently transfected with 5μg of pSC66 or pSC66-F1L-Flag-N using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's specifications, and simultaneously infected with VV65 at an MOI of 5 pfu/cell. The localization of cytochrome c was detected as previously described (9). Briefly, cells were fixed at room temperature for 30 min with 3% paraformaldehyde in PBS. Following fixation, the cells were permeabilized with 0.25% (w/v) saponin (Sigma Chemical Co.) in PBS and incubated on ice for 5 minutes. Glass cover slips were incubated overnight at room temperature with 10μg/mL mouse anti-cytochrome c (clone 6H2.B4) (Pharmingen) followed by the addition of 10μg/mL Alex Fluor 546 goat anti-mouse antibody (Molecular Probes). To detect the Flag-tagged F1L, cells were treated with 10μg/mL anti-Flag M2 monoclonal antibody directly conjugated to FITC (Sigma-Aldrich Inc.). Cellular nuclei and DNA-containing virus factories were identified by staining cells with 1μg/mL Hoechst 33342 (Molecular Probes) at room temperature for 15 minutes. Coverslips were mounted using 50% PBS/50% glycerol solution containing 4mg/mL n-propyl gallate (Sigma Chemical Co.).

Fluorescent images were obtained using a LSM510 laser scanning confocal microscope mounted on a Zeiss Axiovert 100M microscope equipped with a 43 x 1.4 oil immersion Plan-Apochromat objective. EGFP and FITC excitation was induced by illumination at 488nm, and the fluorescent signal was collected using a 505–530nm band pass filter. Alexa-546 and Mitotracker Red CMXRos fluorescence were excited at 543 nm and detected using a 560nm long pass filter. Hoechst 33342 fluorescence was induced by illumination at 364nm, and the fluorescent signal detected using a 385-470nm band pass filter.

Immunoblotting.

Whole cell lysates and fractionated cell lysates were analyzed by electrophoresis on either 8% or 15% SDS polyacrylamide gels. To monitor poly (ADP ribose) polymerase (PARP) cleavage, whole cell lysates were resuspended in SDS loading buffer supplemented with 8M urea and subjected to SDS PAGE. Proteins were transferred to nitrocellulose membranes (Osmonics Inc.) using a Tyler semi-dry transfer apparatus (Tyler Research Instruments) for 2.5 hr at 500 mA. Membranes were blocked for at least 3 hours in PBS containing 0.1% Tween and 5% skim milk. Cytochrome c and PARP were detected using an anti-cytochrome c antibody (clone 7H8.2C12) (Pharmingen) and an anti-PARP antibody (clone C2-10) (Pharmingen) at a dilution of 1:1000 and 1:2000, respectively. Caspase 3 and caspase 9 were detected with polyclonal rabbit anti-caspase 3 and anti-caspase 9 serum at a dilution of 1:5,000 and 1:2,000, respectively (65). All primary antibodies were incubated on the membranes overnight at 4°C. The membranes were probed with either goat anti-mouse HRP-conjugated antibody (Bio-Rad

Laboratories Inc.) at 1:3000 or goat anti-rabbit HRP-conjugated antibody (Bio-Rad Laboratories Inc.) at 1:10000. Proteins were visualized with a chemiluminescent detection system (Amersham Inc.) according to the manufacturer's directions.

3.3 Results

Vaccinia Virus Infection Disrupts the Mitochondrion Component of the Apoptotic Cascade.

The genome of vaccinia virus was completely sequenced in 1990 and it contains no open reading frames with homology to known mitochondrial-localized apoptotic regulators (21). To determine if vaccinia virus encoded a protein(s) that directly inhibited the mitochondrial apoptotic cascade, we initiated our studies by monitoring the ability of vaccinia virus strain Copenhagen, which is devoid of the caspase inhibitor CrmA, to inhibit post-mitochondrial events. Jurkat cells were infected with vaccinia virus strain Copenhagen and apoptosis was induced by staurosporine, a direct activator of the mitochondrial cascade independent of prior caspase activation (8, 56). Post-mitochondrial events were monitored by assessing the cleavage of poly (ADP-ribose) polymerase (PARP), the cleavage of caspases 3 and 9, and cytochrome c release. As shown in Figure 3-1A, Jurkat cells treated with staurosporine rapidly processed full-length PARP protein to its 89 kDa cleaved form. In the presence of vaccinia virus infection, this cleavage event was completely blocked indicating that vaccinia virus was able to inhibit cell death induced by staurosporine (Figure 3-1A). To determine the effect of vaccinia virus infection on the activation of caspases 3 and 9 and the release of cytochrome c, infected Jurkat cells were treated with staurosporine and fractionated into membrane and cytosolic

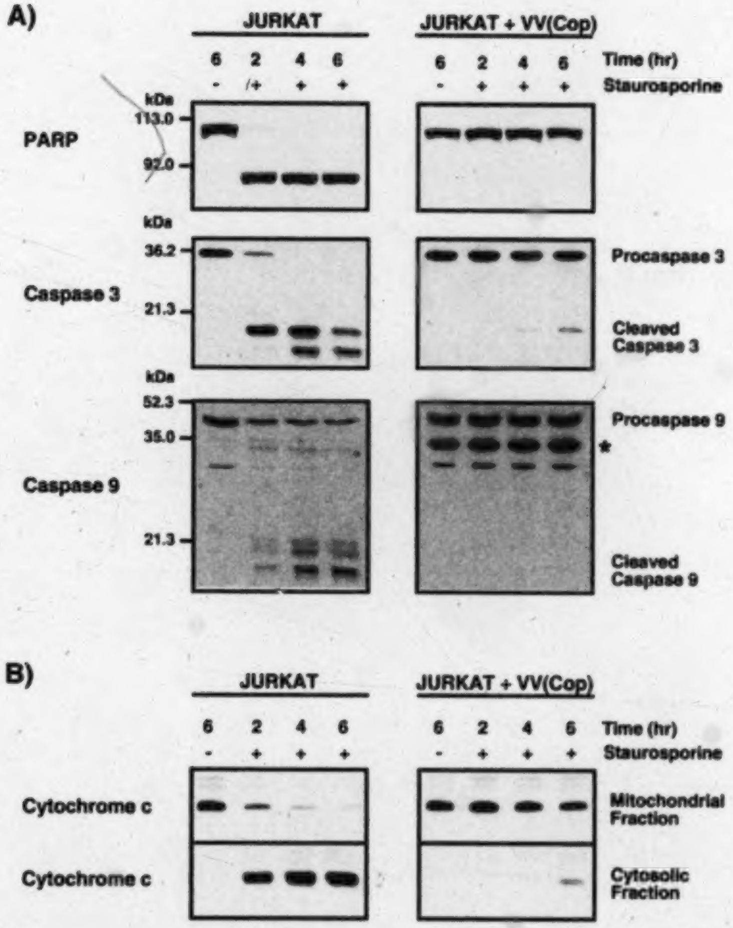

A)
JURKAT
JURKAT + VV(Cop)
6 2 4 6
- + + +
6 2 4 6
- + + +
Time (hr)
Staurosporine
kDa
113.0
92.0
PARP
kDa
36.2
21.3
Caspase 3
Procaspase 3
Cleaved Caspase 3
kDa
52.3
35.0
21.3
Caspase 9
Procaspase 9
*
Cleaved Caspase 9
B)
JURKAT
JURKAT + VV(Cop)
6 2 4 6
- + + +
6 2 4 6
- + + +
Time (hr)
Staurosporine
Cytochrome c
Cytochrome c
Mitochondrial Fraction
Cytosolic Fraction

Figure 3-1 Vaccinia virus strain Copenhagen protects cells from staurosporine-induced apoptosis.

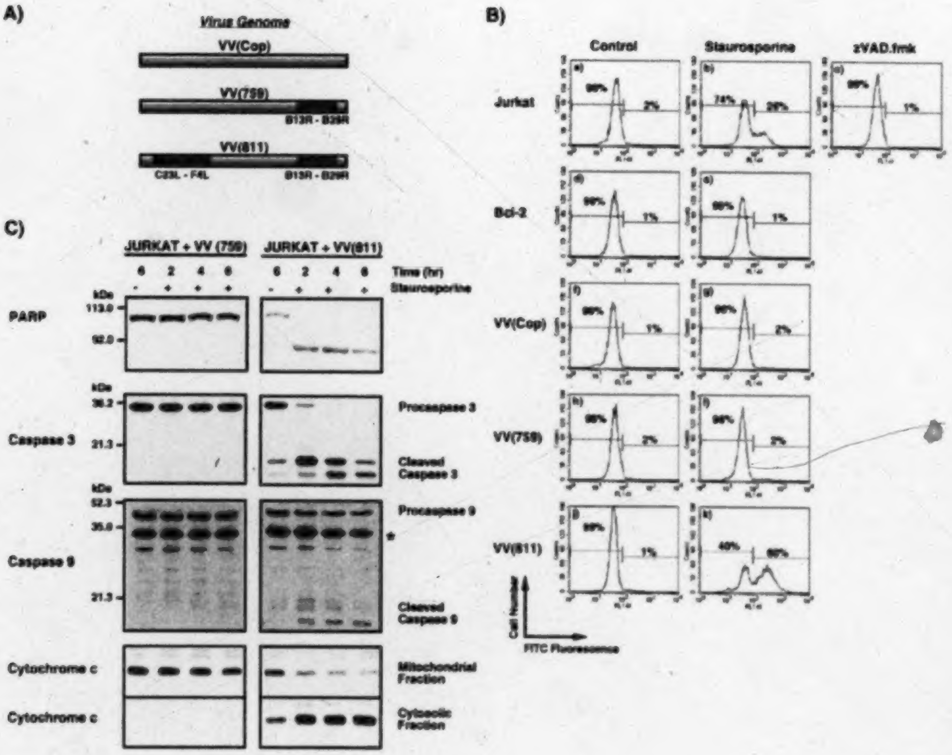
Jurkat cells were mock infected or infected with vaccinia virus strain Copenhagen at an MOI of 10. Five hours post infection, cells were treated with 1 μM staurosporine for 2, 4, and 6 hours. (A) At the indicated times, cell lysates were generated and the cleavage of PARP caspases 3 and 9 were monitored by western blot analysis. * Denotes a cross reacting protein in vaccinia virus infected lysates. (B) To detect cytochrome c release, cells were permeabilized with digitonin and fractionated into mitochondrial-containing membranous fractions and cytosolic fractions. Cytochrome c translocation was assessed by western blot analysis.

fractions followed by western blotting. Mock infected cells treated with staurosporine displayed activation of both caspases 3 and 9 and loss of cytochrome c from the membrane fraction to the cytosolic fraction (Figure 3-1A and B). In contrast, vaccinia virus infection significantly reduced the activation of both caspase 9 and caspase 3 and also prevented the translocation of cytochrome c demonstrating that vaccinia virus infection blocked all post-mitochondrial events tested (Figure 3-1A and B).

Vaccinia Virus Deletion Mutants have Distinguishable Affects on Apoptosis.

To identify the vaccinia virus-encoded gene(s) responsible for inhibiting apoptosis, we utilized two vaccinia virus strain Copenhagen deletion viruses, VV759 and VV811 (45). VV759 is missing 18 open reading frames at the right terminus of the vaccinia virus genome while VV811 lacks a total of 55 open reading frames from both ends of the genome (Figure 3-2A). To determine if the deletion viruses VV759 and VV811 retained the ability to inhibit apoptosis, Jurkat cells were infected, treated with staurosporine, and apoptosis was monitored by assessing the levels of DNA fragmentation. As shown in Figure 3-2B, all untreated cells displayed low levels of DNA fragmentation (panels a, d, f, h, and j). Treatment with staurosporine resulted in 26% of Jurkat cells positive for DNA fragmentation (Figure 3-2B, panel b) which was completely inhibited by pretreatment with the broad-spectrum caspase inhibitor, zVAD.fmk (Figure 3-2B, panel c). Jurkat cells engineered to overexpress Bcl-2 were resistant to staurosporine-induced DNA fragmentation indicating that staurosporine-induced apoptosis occurred through the mitochondrial route as expected (Figure 3-2B, panel e) (8, 56). Infection of Jurkat cells with vaccinia virus strain Copenhagen inhibited DNA

Figure 3-2 Vaccinia virus deletion mutant VV811 is unable to protect cells from staurosporine-induced apoptosis.
(A) Schematic representation of the vaccinia virus strain Copenhagen deletion mutants. VV759 is missing open reading frames B13R to B29R. VV811 is missing open reading frames between B13R to B29R and C23L to F4L. (B) DNA fragmentation is blocked by infection with vaccinia virus strain Copenhagen and VV759 but not VV811. Jurkat cells were infected with vaccinia virus strain Copenhagen, VV759 and VV811 at an MOI of 10. Five hours post infection, cells were treated with 2.5 μM staurosporine for 2 hours and DNA fragmentation was assessed by TUNEL. (a) Untreated Jurkat cells; (b) Jurkat cells treated with staurosporine; (c) Jurkat cells treated staurosporine in the presence of 100 μM zVAD.fmk; (d) Jurkat cells overexpressing Bcl-2; (e) Jurkat cells overexpressing Bcl-2 treated with staurosporine; (f) Jurkat cells infected with vaccinia virus strain Copenhagen; (g) Jurkat cells infected with vaccinia virus strain Copenhagen and treated with staurosporine; (h) Jurkat cells infected with VV759; (i) Jurkat cells infected with VV759 and treated with staurosporine; (j) Jurkat cells infected with VV811; (k) Jurkat cells infected with VV811 and treated with staurosporine. (C) VV759 infection but not VV811 infection protects cells from apoptotic events downstream of the mitochondria. Jurkat cells were infected with VV759 or VV811 at an MOI of 10 and apoptosis was triggered 5 hours post infection with 1 μM staurosporine. At 2, 4 and 6 hours cell lysates were generated and the cleavage of PARP, caspases 3 and 9 were monitored by western blot analysis. * Denotes a cross reacting protein in vaccinia virus infected lysates. To detect cytochrome c release, cells were permeabilized with digitonin and fractionated into mitochondrial-containing membranous fractions and cytosolic fractions. Cytochrome c translocation was assessed by western blot analysis.


A)
Virus Genome
VV(Cop)
VV(759)
B13R - B29R
VV(811)
C23L - F4L
B13R - B29R
B)
Control
Staurosporine
zVAD.fmk
Jurkat
a)
98%
2%
b)
74%
26%
c)
99%
1%
Bcl-2
d)
99%
1%
e)
99%
1%
VV(Cop)
f)
99%
1%
g)
98%
2%
VV(759)
h)
98%
2%
i)
98%
2%
VV(811)
j)
99%
1%
k)
40%
60%
Cell Number
FITC Fluorescence
C)
JURKAT + VV (759)
JURKAT + VV(811)
6
2
4
6
Time (hr)
-
+
+
+
Staurosporine
kDa
113.0
92.0
PARP
36.2
21.3
Caspase 3
Procaspase 3
Cleaved Caspase 3
52.3
35.0
21.3
Caspase 9
Procaspase 9
*
Cleaved Caspase 9
Cytochrome c
Mitochondrial Fraction
Cytochrome c
Cytosolic Fraction

fragmentation (Figure 3-2B, panel g) as did infection of Jurkat cells with VV759 (Figure 3-2B, panel i). Jurkat cells infected with VV811, however, were no longer resistant to staurosporine-induced DNA fragmentation (Figure 3-2B, panel k). In fact, infection with VV811 augmented the level of DNA fragmentation detected following staurosporine treatment (Figure 3-2B, compare panels b and k). These data demonstrate that VV811 is missing the open reading frame(s) necessary for inhibiting the mitochondrial apoptotic cascade.

Our data demonstrated that vaccinia virus strain Copenhagen inhibited post-mitochondrial events such as caspase 9, caspase 3 and PARP cleavage, and blocked release of cytochrome c (Figure 3-1). Therefore, we asked if VV811 was also capable of inhibiting these same post-mitochondrial events. Jurkat cells were infected with either VV759 or VV811, treated with staurosporine, and cellular lysates were subjected to western blot analysis. Similar to infection with vaccinia virus strain Copenhagen (Figure 3-1), infection of cells with VV759 resulted in resistance to staurosporine-induced PARP, caspase 9, and caspase 3 cleavage as well as the inhibition of cytochrome c release into the cytosolic fraction (Figure 3-2C). In contrast, VV811 infection was unable to prevent cleavage of PARP, cleavage of caspases 3 and 9, and the release of cytochrome c (Figure 3-2C). These observations clearly indicated that infection with VV811 was unable to inhibit the mitochondrial-mediated apoptotic pathway, including cytochrome c release, the pivotal step in the cascade (Figure 3-2C). Significantly, even in the absence of staurosporine, VV811 infection resulted in some cleavage of PARP, caspase 3, and caspase 9 as well as release of cytochrome c (Figure 3-2C) indicating that VV811 infection also activated the apoptotic pathway in Jurkat cells.

In addition to releasing pro-apoptotic molecules from the intermembrane space, mitochondria also undergo a loss of the electrochemical gradient across the inner mitochondrial membrane during apoptosis which has been linked to the release of cytochrome c (40, 68). Loss of the inner mitochondrial membrane potential following an apoptotic insult can be monitored by measuring uptake of the potential sensitive mitochondrion-specific dye TMRE (16, 43). To determine if VV811 infection retained the ability to inhibit apoptosis-induced loss of the mitochondrial membrane potential, we infected cells with either vaccinia virus strain Copenhagen, VV759 or VV811 and assessed the levels of TMRE uptake by flow cytometry. In the absence of staurosporine, all cells demonstrated the ability to efficiently take up TMRE indicating preservation of the inner mitochondrial membrane potential (Figure 3-3, panel a, e, g, i, k). Following treatment with staurosporine, 76% of Jurkat cells displayed reduced levels of TMRE fluorescence (Figure3-3, panel b). As a control, mock infected cells were also treated with the membrane uncoupler ClCCP which triggers loss of the inner mitochondrial membrane potential resulting in an overall reduction of TMRE uptake (Figure 3-3, panel c). Pre-treatment with zVAD.fmk did not affect the levels of TMRE uptake following treatment with staurosporine indicating that loss of the inner mitochondrial membrane potential was a caspase-independent event as previously documented (Figure 3-3, panel d) (8, 56). As expected, the majority of cells overexpressing Bcl-2 maintained the ability to uptake TMRE following staurosporine treatment (Figure 3-3, panel f). Compared to uninfected cells, 67% and 76% of vaccinia virus strain Copenhagen- and VV759-infected cells demonstrated efficient uptake of TMRE following the addition of staurosporine (Figure 3-3, panel h and j). In contrast, only 20% of cells infected with VV811 were able

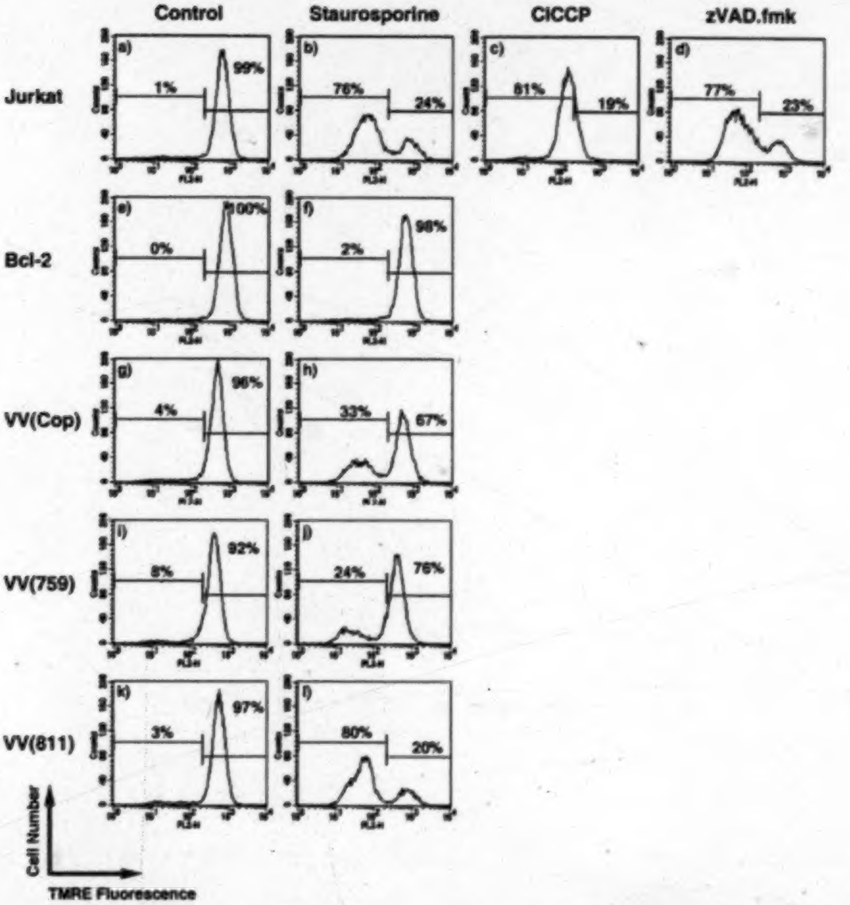


Figure 3-3 Vaccinia virus deletion mutant VV811 is unable to protect cells from staurosporine-induced disruption of the inner mitochondrion membrane potential. Jurkat cells were mock infected or infected with vaccinia virus strain Copenhagen, VV759 or VV811 at an MOI of 10. Five hours post infection cells were treated with 1 μM staurosporine for 1 hour. Loss of the inner mitochondrial membrane potential was monitored by TMRE fluorescence. (a) Untreated Jurkat cells; (b) Jurkat cells treated with staurosporine; (c) Jurkat cells treated with the membrane uncoupler ClCCP; (d) Jurkat cells treated with staurosporine in the presence of 100 μM zVAD.fmk; (e) Jurkat cells overexpressing Bcl-2; (f) Jurkat cells overexpressing Bcl-2 and treated with staurosporine; (g) Jurkat cells infected with vaccinia virus strain Copenhagen; (h) Jurkat cells infected with vaccinia virus strain Copenhagen and treated with staurosporine; (i) Jurkat cells infected with VV759; (j) Jurkat cells infected with VV759 and treated with staurosporine; (k) Jurkat cells infected with VV811; (l) Jurkat cells infected with VV811 and treated with staurosporine.

to efficiently take up TMRE indicating that VV811 infection was unable to maintain the mitochondrial membrane potential (Figure 3-3, panel I). Thus, cells infected with VV811 were incapable of inhibiting all aspects of apoptosis previously inhibited by infection with vaccinia virus strain Copenhagen.

F1L Protects VV811 Infected Cells from Staurosporine- and Fas-induced Loss of the Inner Mitochondrial Membrane Potential.

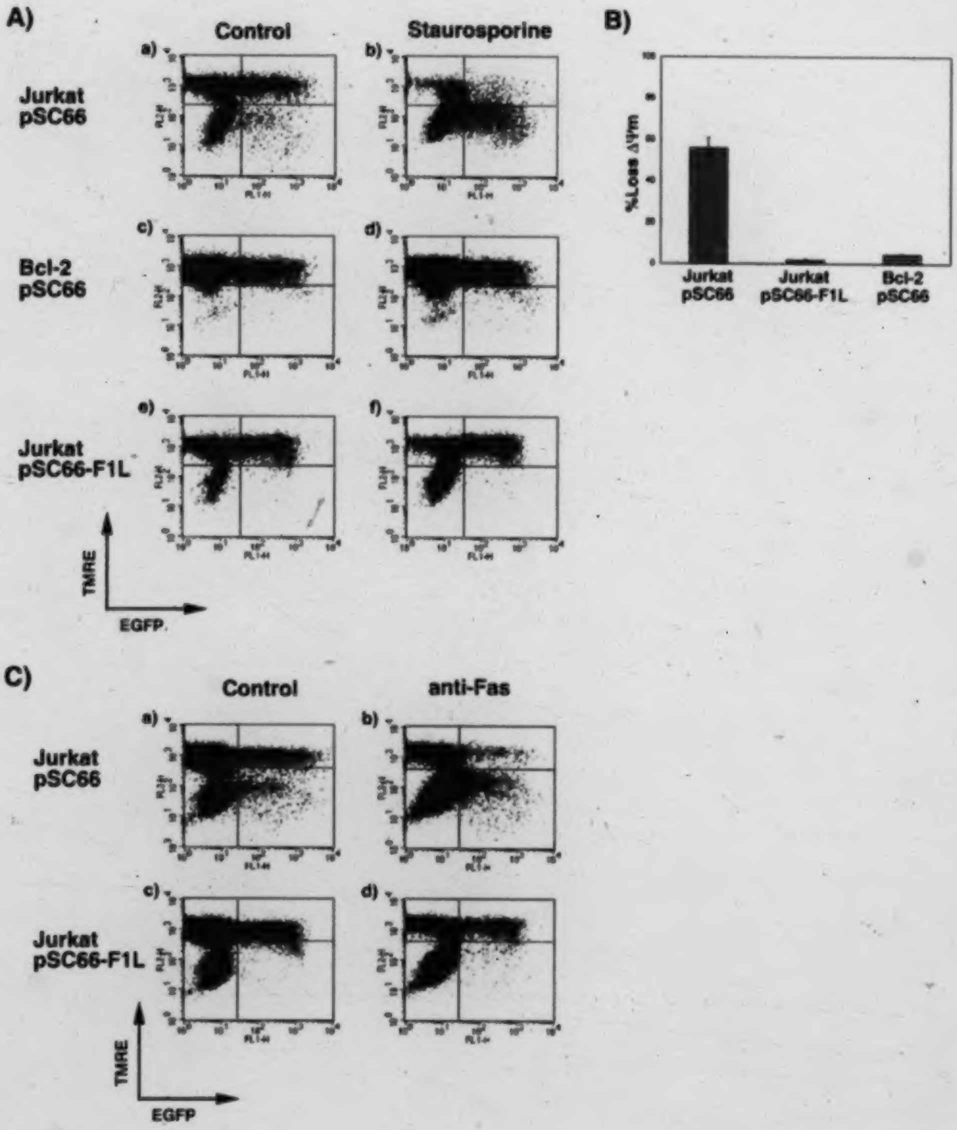
The inability of VV811 to inhibit the release of cytochrome c and loss of the mitochondrial membrane potential suggested at least one of the open reading frames absent in this virus was integral to the inhibition of mitochondrial dependent apoptosis. Since VV811 is missing approximately 55 open reading frames, we sought to exclude additional open reading frames that likely did not function at the mitochondria to inhibit apoptosis. To further narrow our search for candidate open reading frames, we performed a peptide-based blast search screening for vaccinia virus open reading frames that contained putative C-terminal mitochondrial targeting motifs present in anti-apoptotic proteins including M11L, Bcl-2, and Bcl-XL (1, 17). Using this strategy, we identified one vaccinia virus open reading frame, F1L, for further analysis.

Since VV811 infection was unable to inhibit apoptosis, we utilized VV811 and asked if F1L could restore the inhibition of apoptosis. Jurkat cells were infected with VV811 and co-transfected with pSC66-EGFP in combination with either pSC66-F1L or pSC66. Infection with VV811, which is unable to inhibit apoptosis, drives the expression of both F1L and EGFP which are placed under the control of a poxvirus promoter in pSC66, and EGFP expression serves as a reporter for infected and transfected cells. To ensure that the majority of EGFP positive cells were expressing F1L, a ratio of 4μg:1μg (pSC66-F1L:pSC66-EGFP) was used for co-transfection and apoptosis was monitored by assessing TMRE uptake in the EGFP positive population. All infected and co-transfected cells showed a population of cells that were positive for both EGFP expression and TMRE (Figure 3-4A, panels a, c and e). Following treatment with staurosporine, cells infected with VV811 and co-transfected with pSC66 and pSC66-EGFP demonstrated a population of cells with decreased ability to take up TMRE indicative of cells dying by apoptosis (Figure 3-4A panel b). Efficient TMRE uptake occurred in VV811 infected, pSC66-EGFP and pSC66 co-transfected cells overexpressing Bcl-2 following treatment with staurosporine (Figure 3-4A panel d). This result indicated that Bcl-2 expression could replace the anti-apoptotic protein(s) missing in VV811. Most importantly, cells infected with VV811 and co-transfected with pSC66-EGFP and pSC66-F1L, were competent for TMRE uptake in the presence of staurosporine (Figure 3-4A, panel f), and the results of three independent experiments are graphically represented in Figure 3-4B. These results demonstrated that the expression of F1L during VV811 infection could restore the ability of VV811 to maintain the inner mitochondrial membrane potential induced by treatment with pro-apoptotic stimuli.

Since our previous data showed that infection with vaccinia virus strain Copenhagen inhibited the Fas-mediated apoptotic cascade, we next asked whether the expression of F1L could restore the ability of VV811 to inhibit Fas-induced death (65). To test this, Jurkat cells were infected with VV811 and co-transfected with pSC66-EGFP in combination with pSC66 or pSC66-F1L, and apoptosis was induced by the addition of anti-Fas antibody. Jurkat cells infected with VV811 and co-transfected with pSC66-

Figure 3-4 The vaccinia virus F1L open reading frame restores the ability of VV811 to maintain the inner mitochondrion membrane potential.

Jurkat cells were infected with VV811 and transfected with 1 µg of the reporter plasmid pSC66-EGFP in combination with 4 µg of pSC66 or pSC66-F1L. (A) At five hours post infection/transfection, cells were treated with 1 µM staurosporine for 2 hours and the inner mitochondrial membrane potential was assessed by TMRE fluorescence. (a) Untreated Jurkat cells infected with VV811 and transfected with pSC66-EGFP and pSC66; (b) Jurkat cells infected with VV811 and transfected with pSC66-EGFP and pSC66 and treated with staurosporine; (c) Jurkat cells overexpressing Bcl-2 infected with VV811 and transfected with pSC66-EGFP and pSC66; (d) Jurkat cells overexpressing Bcl-2 infected with VV811 and transfected with pSC66-EGFP and pSC66 and treated with staurosporine; (e) Jurkat cells infected with VV811 and transfected with pSC66-EGFP and pSC66-F1L; (f) Jurkat cells infected with VV811 and transfected with pSC66-EGFP and pSC66-F1L and treated with staurosporine. (B) Graphical representation of the experiment described in (A). Standard deviations were calculated from three independent experiments. (C) Following infection and transfection, cells were treated with 250 ng/mL anti-Fas and 5 µg/ml cycloheximide for 8 hours and the mitochondrial membrane potential was assessed by TMRE fluorescence. (a) Untreated Jurkat cells infected with VV811 and transfected with pSC66-EGFP and pSC66; (b) Jurkat cells infected with VV811 and transfected with pSC66-EGFP and pSC66 and treated with anti-Fas; (c) Jurkat cells infected with VV811 and transfected with pSC66-EGFP and pSC66-F1L; (d) Jurkat cells infected with VV811 and transfected with pSC66-EGFP and pSC66-F1L and treated with anti-Fas.


A)
Control
Staurosporine
a)
b)
Jurkat pSC66
c)
d)
Bcl-2 pSC66
e)
f)
Jurkat pSC66-F1L
FL2-H
FL1-H
TMRE
EGFP
B)
%Loss ΔΨm
100
80
60
40
20
0
Jurkat pSC66
Jurkat pSC66-F1L
Bcl-2 pSC66
C)
Control
anti-Fas
a)
b)
Jurkat pSC66
c)
d)
Jurkat pSC66-F1L
TMRE
EGFP

EGFP and pSC66 showed a decreased ability to uptake TMRE following anti-Fas treatment (Figure 3-4C, panel b). In contrast, cells co-transfected with pSC66-EGFP and pSC66-F1L were competent for TMRE uptake following anti-Fas treatment indicating that F1L was capable of inhibiting loss of the mitochondrial membrane potential induced by activation of the Fas receptor (Figure 3-4C, panel d).

The Vaccinia Virus F1L Protein Localizes to Mitochondria.

Since our data showed that vaccinia virus infection blocked apoptosis by interfering with both cytochrome c release and loss of the inner mitochondrial membrane potential, we were prompted to address the cellular localization of F1L. To determine the localization of F1L, an N-terminal EGFP-F1L fusion was generated and localization was visualized by confocal microscopy. HeLa cells were transfected with either pEGFP or pEGFP-F1L and mitochondria were labeled with Mitotracker Red, a mitochondrion specific dye (67). Transfection of HeLa cells with EGFP alone resulted in a diffuse fluorescence signal throughout the cell (Figure 3-5A, panel a) which did not co-localize with Mitotracker Red (Figure 3-5A, panel c). In contrast, cells transfected with EGFP-F1L displayed a punctate staining pattern with a distribution similar to Mitotracker Red (Figure 3-5A, panel d and e). When the two images were superimposed, a uniform yellow image was produced indicative of two co-incident fluorescent signals and demonstrating that F1L localized to the mitochondria (Figure 3-5A, panel f).

To investigate the localization of F1L during virus infection we generated an N-terminal Flag-tagged version of F1L under the control of a poxviral promoter, pSC66-F1L-Flag-N. HeLa cells were infected with vaccinia virus and transfected with pSC66-

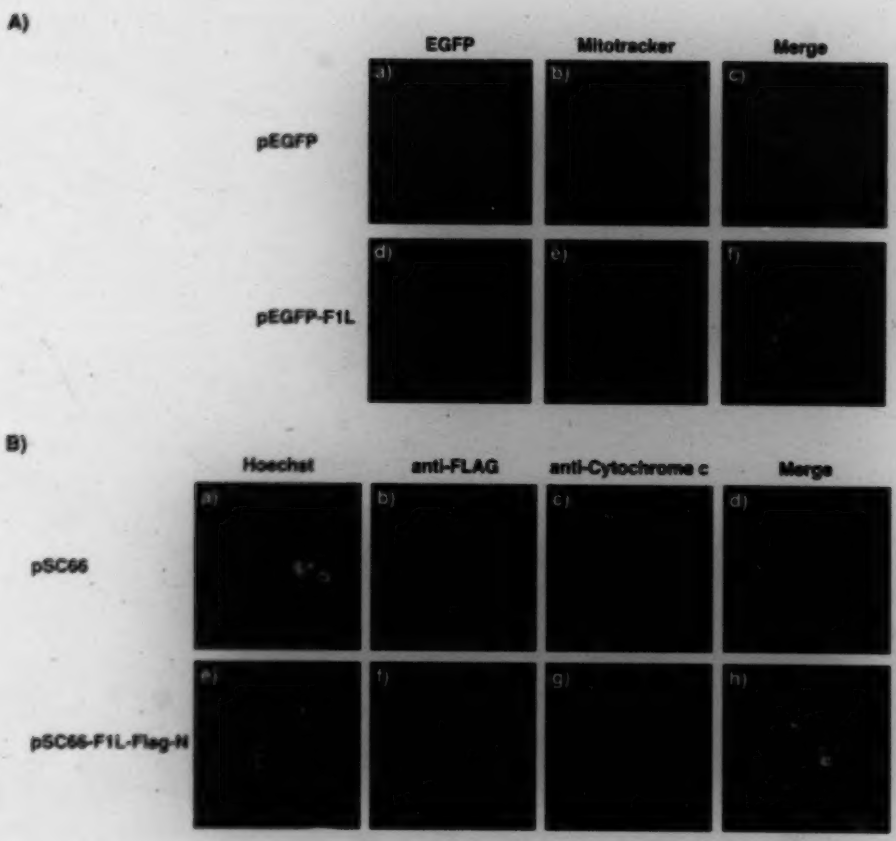


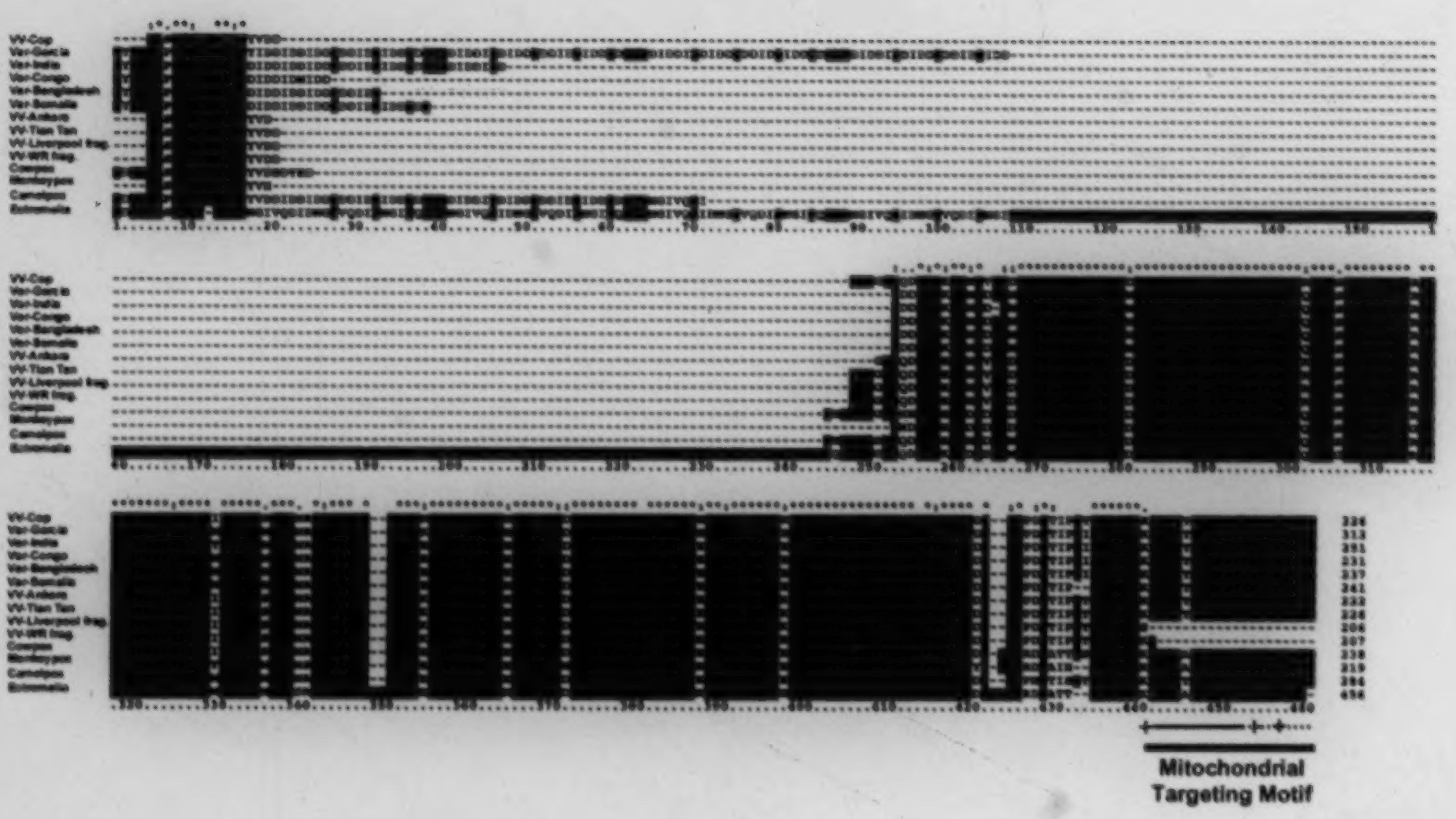
Figure 3-5 The product of the vaccinia virus F1L open reading frame localizes to mitochondria.
(A) Live HeLa cells were transiently transfected with either pEGFP (panel a) or an EGFP tagged version of F1L, pEGFP-F1L (panel d) and visualized by confocal microscopy. Mitochondria were labeled with the mitochondrion specific dye MitotrackerRed CMXRos (panels b and e). Merged images (panels c and f) indicate that EGFP-F1L localizes to the mitochondria whereas EGFP does not. (B) HeLa cells were infected with vaccinia virus strain Copenhagen at an MOI of 3 and transfected with pSC66 or pSC66-F1L-Flag-N. At 8 hours post infection cells were fixed and permeabilized. Mitochondria were labeled using an anti-cytochrome c antibody followed by detection with the Alexa Fluor 546 conjugated goat-anti-mouse antibody (panels c and g). The nucleus and DNA containing viral factories were labeled with Hoechst (panels a and e). The localization of Flag tagged F1L was visualized with a FITC conjugated mouse-anti-Flag antibody (panels b and f). Merged images from panels (f) and (g) indicate that F1L-Flag-N localized to the mitochondria during vaccinia virus infection (panel h).

F1L-Flag-N or pSC66 alone. This ensured that only cells infected with vaccinia virus and transfected would be positive for Flag-F1L expression. In infected and transfected HeLa cells, Hoechst staining detected both the nucleus and DNA containing virus factories adjacent to the nucleus (Figure 3-5B panel a and e). Using an antibody specific for the Flag tag, no Flag-F1L was detected in cells infected and transfected with pSC66 alone (Figure 3-5B panel b). In contrast, cells infected and transfected with the Flag-F1L construct demonstrated a staining pattern typical of mitochondrial localization (Figure 3-5B panel f). In addition, Flag-F1L co-localized with mitochondria that were visualized by staining with an antibody specific for cytochrome c (Figure 3-5B panel g and h). Our results provide clear evidence that F1L localizes to mitochondria in the absence and presence of infection.

The C-terminal Hydrophobic Domain of F1L is Critical for Mitochondrial Localization.

The poxvirus family, of which vaccinia virus is the prototypical member, consists of a large family of viruses that infect both vertebrates and invertebrates. To date, only members of the Orthopoxvirus genus, of which vaccinia virus is a member, contain F1L open reading frames (Figure 3-6). The greatest sequence diversity among the various F1L orthologs is located within the N-terminal region, with strains of variola virus, camelpox virus and ectromelia virus displaying a series of unique repeats (Figure 3-6). Sequence analysis revealed no homology between F1L and members of the Bcl-2 family of proteins that normally regulate mitochondria during apoptosis. In all F1L orthologs, sequence analysis demonstrated the presence of a C-terminal hydrophobic domain, amino acids

Figure 3-6 F1L is highly conserved within the Orthopoxvirus genus.
Alignment of F1L orthologs encoded by members of the Orthopoxvirus genus generated with the ClustalX alignment program. Conserved residues are highlighted in green. '*' indicates fully conserved residues; ':' indicates strongly conserved residues; '.' Indicates weakly conserved residues. The mitochondrial targeting motif, underlined in red, is conserved in all the orthologs. '+' indicates positive charge amino acids in the mitochondrial targeting motif; solid black line indicates the proposed transmembrane domain; black dotted line indicates the positive charged tail. VV-Cop, vaccinia virus strain Copenhagen (F42506); Var-Garcia, variola virus strain Garcia (G72153); Var-India, variola virus strain India (E36839); Var-Congo, variola virus strain Congo (AAA69330); Var-Bangladesh, variola virus strain Bangladesh (AAA60773); Var-Somalia, variola virus strain Somalia (AAA69436); VV-Ankara, vaccinia virus strain Ankara (AAB96412); VV-Tian Tan, vaccinia virus strain Tian Tan (AAF33891); VV-Liverpool frag., vaccinia virus strain Liverpool fragment (AAA48297); VV-WR, vaccinia virus strain Western Reserve fragment (I36213); Cowpox strain Gri-90 (CAA64115); Monkeypox strain Zaire (NP_536460); Camelpox strain M-96 (AAL73743); Ectromelia virus strain Moscow (NP_671543).


VV-Cop
Var-Garcia
Var-India
Var-Congo
Var-Bangladesh
Var-Somalia
VV-Ankara
VV-Tian Tan
VV-Liverpool frag.
VV-WR frag.
Cowpox
Monkeypox
Camelpox
Ectromelia
Mitochondrial Targeting Motif

207-218, flanked by positively charged residues and a C-terminal short hydrophilic tail (Figure 3-6). Since a growing number of proteins, including some members of the Bcl-2 family, localize to membranes through a C-terminal transmembrane domain, the presence of this conserved C-terminal domain within F1L suggested that the domain might serve as a mitochondrion targeting sequence (28, 44).

To determine if the C-terminal domain of F1L was necessary for mitochondrial localization we generated an EGFP version of F1L, pEGFP-F1L(1-206), lacking the final twenty amino acids encompassing the hydrophobic domain and the short hydrophilic tail. HeLa cells transfected with pEGFP-F1L(1-206) demonstrated EGFP fluorescence throughout the cytoplasm and nucleus (Figure 3-7 panel a) which was clearly distinct from the mitochondrial localization pattern of Mitotracker Red (Figure 3-7 panels b and c). This result suggested that the C-terminal domain of F1L played a critical role in mitochondrial localization. To determine if the C-terminal domain was sufficient for mitochondrial localization we appended the C-terminal fragment of F1L onto EGFP to create pEGFP-F1L(199-226). HeLa cells transfected with pEGFP-F1L(199-226) showed localization that mirrored the mitochondrion staining by Mitotracker Red (Figure 3-7 panels d and e). When the fluorescent signals of EGFP-F1L(199-226) and Mitotracker Red were superimposed, co-localization was evident clearly demonstrating that a mitochondrial targeting sequence was contained within amino acids 199-226 of F1L (Figure 3-7 panel f).

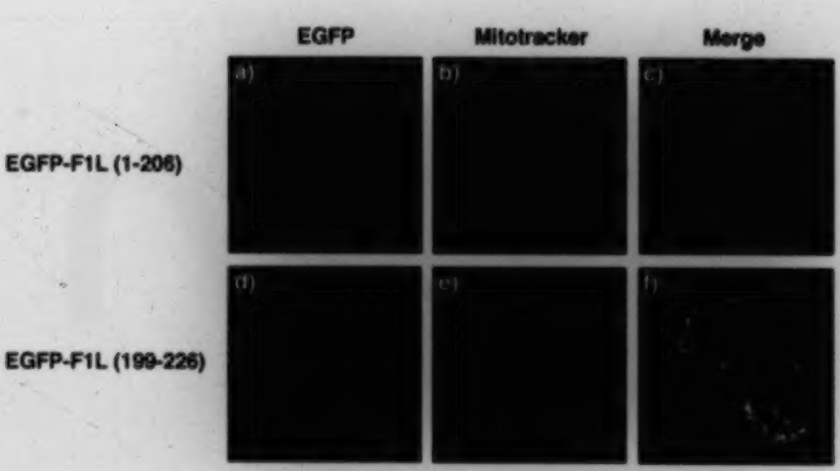


Figure 3-7 The C-terminal hydrophobic domain of F1L is necessary and sufficient for mitochondria localization.

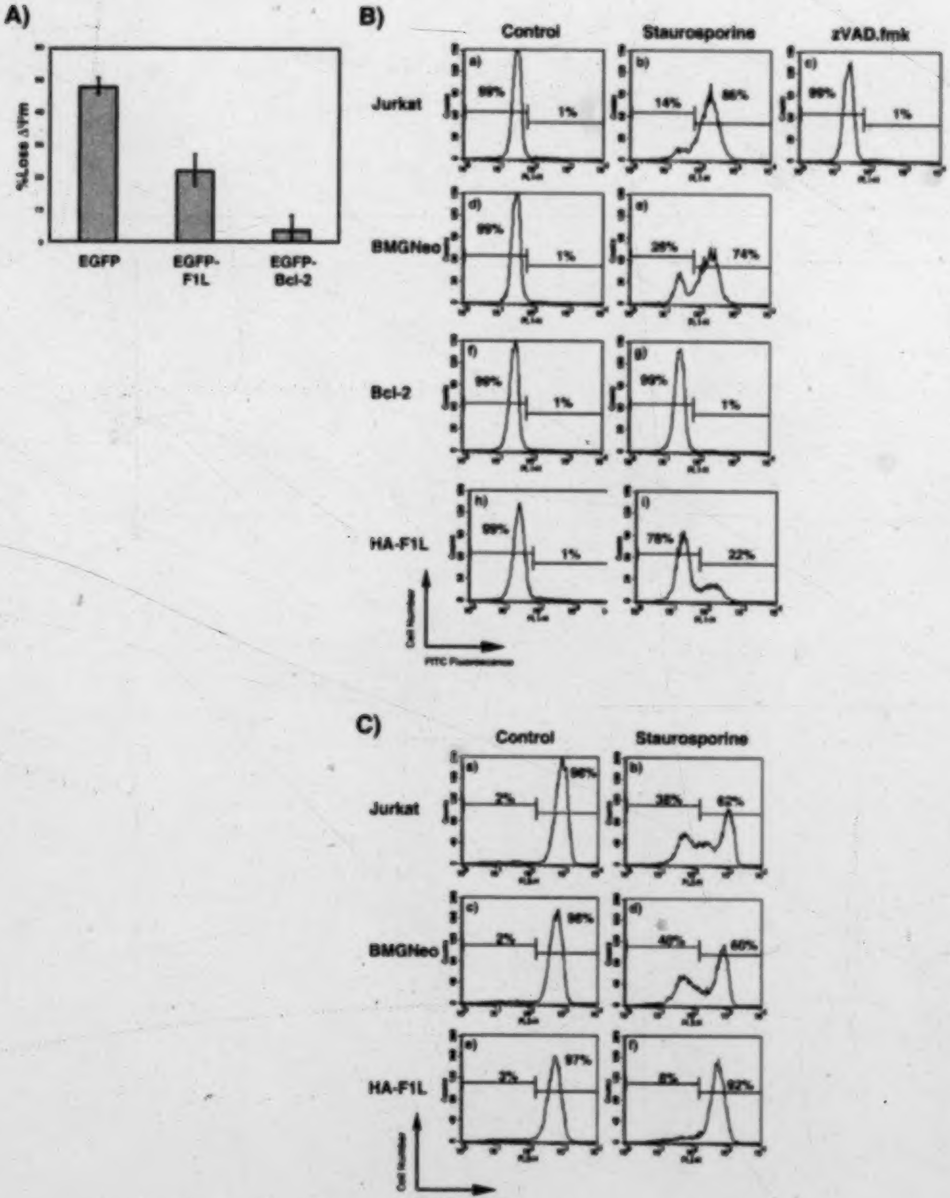
HeLa cells were transfected with a truncated version of F1L lacking the last 20 amino acids, pEGFP-F1L(1-205) or with pEGFP-F1L(199-226) containing the C-terminal region of F1L and F1L localization was visualized by confocal microscopy. Mitochondria were labeled with the mitochondrion specific dye MitotrackerRed CMXRos (panels b and e). Merging of panels (a) and (b) indicated that EGFP-F1L(1-205) did not localize with Mitotracker Red (panel c) whereas pEGFP-F1L(199-226) (panel d) localized to the mitochondria as indicated by the merged image (panel f).

F1L Expression is Sufficient to Block Loss of the Inner Mitochondrion Membrane Potential.

Our results showed that F1L localized to mitochondria in the presence and absence of infection and restored the anti-apoptotic function during VV811 infection. Therefore, we next asked if F1L could function in the absence of virus infection. HeLa cells were transiently transfected with either pEGFP, pEGFP-Bcl-2 or pEGFP-F1L and the loss of the inner mitochondrial membrane potential was monitored by TMRE fluorescence following an apoptotic stimulus. As shown in Figure 3-8A, greater than 40% of the EGFP-transfected cells demonstrated loss of the inner mitochondrial membrane potential following treatment with anti-Fas. In contrast, transient expression of EGFP-Bcl-2 significantly inhibited loss of the inner mitochondrial membrane potential (Figure 3-8A). Expression of EGFP-F1L also prevented the loss of the mitochondrial membrane potential as compared to cells expressing EGFP (Figure 3-8A).

To further support our observations we generated Jurkat cells stably expressing an HA-tagged version of F1L and assessed resistance to apoptosis. Clones positive for the F1L transcript were identified by reverse transcriptase PCR (RT-PCR) (Appendix Figure A-1). We first treated the stable cell lines with staurosporine and monitored DNA fragmentation. In the absence of staurosporine, less than 1% of the cells demonstrated fragmented DNA (Figure 3-8B panels a, d, f and h). Following treatment with staurosporine, Jurkat cells and Jurkat cells transfected with the empty vector, BMGneo, showed 86% and 74% of the cells positive for DNA fragmentation and pre-treatment with zVAD.fmk completely inhibited DNA fragmentation (Figure 3-8B panel b, c and e). Jurkat cells expressing Bcl-2 were protected from staurosporine-induced DNA

Figure 3-8 F1L expression in the absence of virus infection maintains the inner mitochondrial membrane potential following apoptosis.
(A) EGFP-tagged F1L inhibits loss of the inner mitochondrial membrane potential. HeLa cells were transfected with pEGFP, pEGFP-Bcl-2 or pEGFP-F1L and treated with 2 μM staurosporine for 2.5 hours. Loss of the inner mitochondrial membrane potential was quantified by TMRE fluorescence. Standard deviations were generated from three independent experiments. (B) F1L protects cells from DNA fragmentation. Jurkat cells were treated with 2 μM staurosporine for 2.5 hours, and DNA fragmentation was assessed using the TUNEL assay. (a) Untreated Jurkat cells; (b) Jurkat cells treated with staurosporine; (c) Jurkat cells treated with staurosporine in the presence of 100 μM zVAD.fmk; (d) Untreated Jurkat cells transfected with the empty vector BMGneo; (e) Jurkat BMGneo cells treated with staurosporine; (f) Jurkat cells overexpressing Bcl-2; (g) Jurkat cells overexpressing Bcl-2 and treated with staurosporine; (h) Jurkat cells stably expressing F1L; (i) Jurkat cells stably expressing F1L and treated with staurosporine. (C) F1L inhibits disruption of the mitochondrial membrane potential. Jurkat cells were treated with 1 μM staurosporine for 1 hour, and the loss of the membrane potential was determined using TMRE fluorescence. (a) Untreated parental Jurkat cells; (b) Jurkat cells treated with staurosporine; (c) Untreated Jurkat cells transfected with the empty vector BMGneo; (d) BMGneo cells treated with staurosporine; (e) Jurkat cells stably expressing F1L; (f) Jurkat cells stably expressing F1L and treated with staurosporine.


A)
%Loss ΔΨm
EGFP
EGFP-F1L
EGFP-Bcl-2
B)
Control
Staurosporine
zVAD.fmk
Jurkat
BMGNeo
Bcl-2
HA-F1L
a)
99%
1%
b)
14%
86%
c)
99%
1%
d)
99%
1%
e)
26%
74%
f)
99%
1%
g)
99%
1%
h)
99%
1%
i)
78%
22%
Cell Number
FITC Fluorescence
C)
Control
Staurosporine
Jurkat
BMGNeo
HA-F1L
a)
2%
98%
b)
38%
62%
c)
2%
98%
d)
40%
60%
e)
2%
97%
f)
8%
92%
Cell Number
TMRE Fluorescence

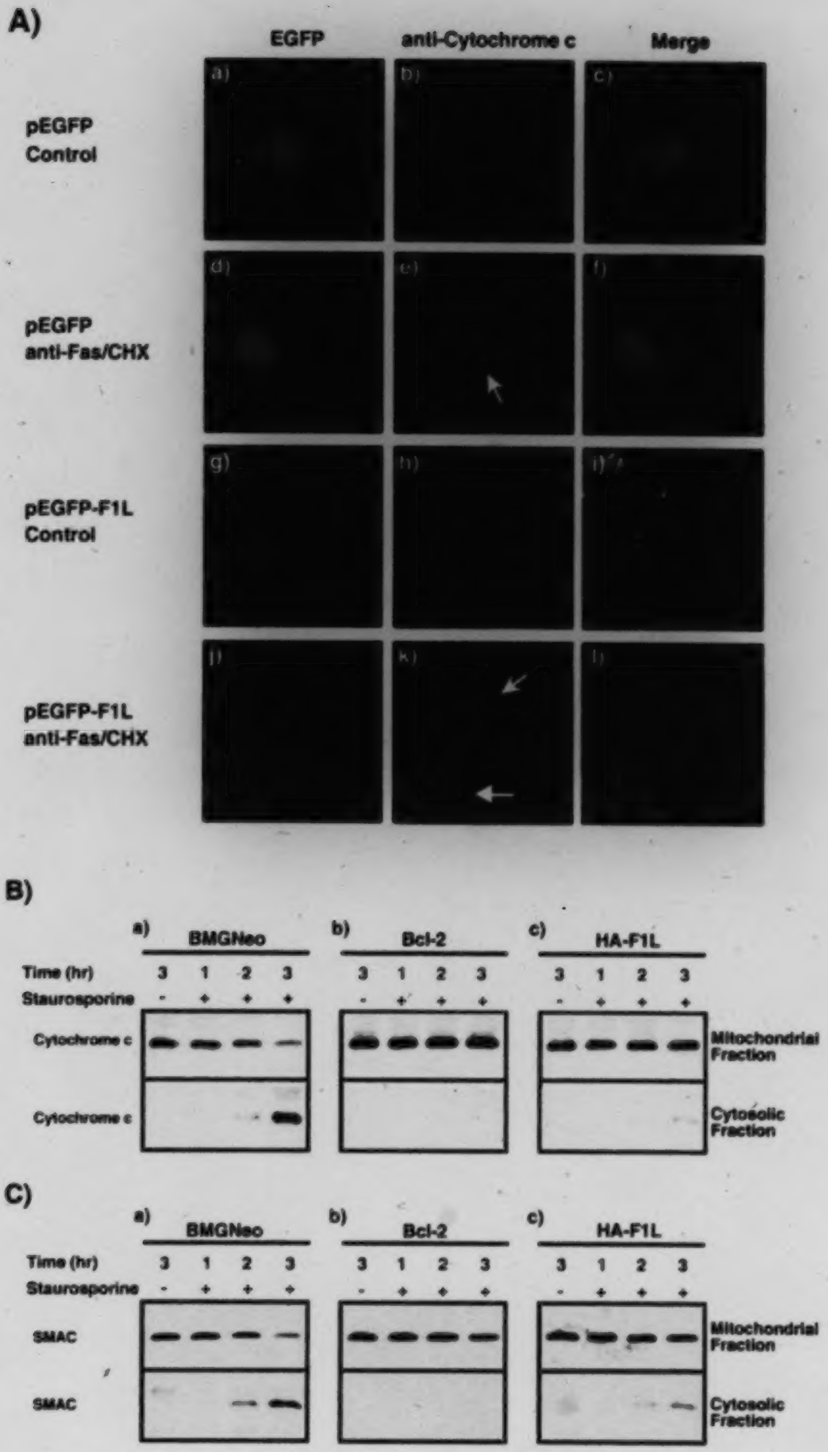
fragmentation (Figure 3-8B panel g). Additionally, Jurkat cells expressing F1L were also resistant to staurosporine-induced apoptosis with only 22% of the cells showing DNA fragmentation (Figure 3-8B panel i) indicating that F1L expression was sufficient to inhibit DNA fragmentation. To confirm that F1L expression inhibited the loss of the mitochondrial membrane potential following treatment with a pro-apoptotic stimulus, we treated the stable clones with staurosporine and monitored loss of the inner mitochondria membrane potential by TMRE uptake (Figure 3-8C). As shown in Figure 3-8C, in the absence of staurosporine all cells displayed high levels of TMRE fluorescence (Figure 3-8C panels a, c and e). Treatment of Jurkat cells or BMGneo stable clones with staurosporine resulted in 38% and 40% of the cells showing decreased uptake of TMRE, respectively (Figure 3-8C panels b and d). Importantly, Jurkat cells expressing F1L maintained high levels of TMRE fluorescence following the addition of staurosporine (Figure 3-8C panel f). In total, ten F1L and ten empty vector clones were assayed for resistance to the loss of the inner mitochondrial membrane potential (Appendix Table A-1). Following staurosporine treatment, eight of the ten clones F1L clones inhibited loss of the inner mitochondrial membrane potential. Taken together, this data demonstrated that F1L functions independently of other vaccinia virus proteins to inhibit the loss of the inner mitochondrion membrane potential.

F1L Inhibits Apoptosis by Blocking Release of Cytochrome c and SMAC.

Anti-apoptotic members of the Bcl-2 family regulate apoptosis by inhibiting the release of pro-apoptotic proteins from the mitochondria (24, 64). Although, F1L displays no sequence similarity to members of the Bcl-2 family, its presence at the mitochondrion suggested that F1L might regulate apoptosis by interfering with the release of pro-apoptotic proteins such as cytochrome c and SMAC. To determine if F1L expression could inhibit cytochrome c release, HeLa cells were transfected with pEGFP or pEGFP-F1L, treated with anti-Fas, and cytochrome c release was detected with an antibody specific for cytochrome c by confocal microscopy. In the absence of anti-Fas, confocal microscopy revealed a punctate distribution of cytochrome c indicative of mitochondrial localized cytochrome c (Figure 3-9A panels b and h). Following the addition of anti-Fas, cells transfected with pEGFP showed a diffuse cytosolic pattern of cytochrome c staining indicating that cytochrome c was released from the mitochondria (Figure 3-9A panel e and f). In contrast, we routinely found that cytochrome c was retained within the mitochondria only in cells transfected with pEGFP-F1L following anti-Fas treatment (Figure 3-9A panel k and i). In fact, surrounding cells not expressing EGFP-F1L showed cytochrome c release and membrane blebbing (Figure 3-9A panel k and i). These observations were confirmed through biochemical analysis of cytochrome c release. Figure 3-9B demonstrates that translocation of cytochrome c from the mitochondria occurred in Jurkat cells at 2 and 3 hours following treatment with staurosporine (Figure 3-9B panel a) whereas cytochrome c release was blocked in cells overexpressing Bcl-2 and reduced in cells expressing F1L (Figure 3-9B panel b and c).

To determine if F1L expression also inhibited the release of SMAC, the mitochondria and cytosolic fractions were immunoblotted with an antibody specific for SMAC (Figure 3-9C). Similar to cytochrome c release, we observed that Jurkat cells treated with staurosporine released SMAC from the mitochondria (Figure 3-9C panel a) while staurosporine-induced SMAC release was blocked by the overexpression of Bcl-2

Figure 3-9 F1L inhibits cytochrome c and SMAC release from mitochondria. (A) HeLa cells were transfected with pEGFP (panel a, b, c, d, e, and f) or with pEGFP-F1L (panel g, h, i, j, k and l). Transfected cells were treated with anti-Fas and cytochrome c was detected using anti-cytochrome c and Alex 546 secondary antibody. DNA was stained with Hoechst to identify nuclei (panels c, f, i and l). Untreated transfected cells showed mitochondrial localization of cytochrome c (panels b, c, h and i) denoted by “ ← “. Following treatment with anti-Fas, EGFP transfected cells showed release of cytochrome c (panel e and f). Cytochrome c was retained in cells transfected with EGFP-F1L (panel k and i) denoted by “ ←“. (B) and (C) F1L expression inhibits cytochrome c and SMAC release. Jurkat cells transfected with the BMGneo empty vector (panels a), overexpressing Bcl-2 (panels b) or expressing F1L (panels c) were treated with 1 μM staurosporine for 1, 2, or 3 hours. To detect cytochrome c and SMAC release, cells were fractionated into mitochondrial-containing membranous fractions and cytosolic fractions. The translocation of cytochrome c and SMAC were monitored by western blot analysis.


A)
EGFP
anti-Cytochrome c
Merge
pEGFP Control
pEGFP anti-Fas/CHX
pEGFP-F1L Control
pEGFP-F1L anti-Fas/CHX
a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
k)
l)
B)
a) BMGNeo
b) Bcl-2
c) HA-F1L
Time (hr) 3 1 2 3
Staurosporine - + + +
Cytochrome c
Cytochrome c
Mitochondrial Fraction
Cytosolic Fraction
C)
a) BMGNeo
b) Bcl-2
c) HA-F1L
Time (hr) 3 1 2 3
Staurosporine - + + +
SMAC
SMAC
Mitochondrial Fraction
Cytosolic Fraction

and reduced in cells expressing F1L (Figure 3-9C panel b and c). Our data demonstrate that the F1L gene product from vaccinia virus localizes to the mitochondrion where it inhibits apoptosis by regulating the loss of the inner membrane potential and the release of the pro-apoptotic proteins SMAC and cytochrome c.

F1L Expression Protects Cells from VV811-induced Apoptosis.

Apoptosis can be triggered by a variety of extrinsic and intrinsic signals (26). Very early in our studies we observed that infection of Jurkat cells with VV811 alone resulted in some cleavage of PARP, caspases 3 and 9 and most notably the translocation of cytochrome c (Figure 3-2C). Although we failed to detect DNA fragmentation after 7 hours of infection, we did observed that infection with VV811 augmented DNA fragmentation following staurosporine treatment (Figure 3-2B). These observations suggested that in the absence of F1L, VV811 could be stimulating an apoptotic response. Therefore, to determine if VV811 infection resulted in apoptosis we infected Jurkat cells with either vaccinia virus strain Copenhagen, VV759, or VV811 for 15 hours, a longer time period than our initial experiments, and quantified DNA fragmentation. Fifteen hours post infection, low levels of DNA fragmentation were detected in uninfected Jurkat cells as well as cells infected with vaccinia virus and VV759 (Figure 3-10A panel a, c and e) whereas Jurkat cells infected with VV811 showed 44% of the cells positive for DNA fragmentation which was inhibited by pretreatment of the cells with zVAD.fmk (Figure 3-10A panel g and h). VV811-induced DNA fragmentation was also inhibited by infecting cells that overexpressed Bcl-2 indicating that apoptosis occurred exclusively through the mitochondrial pathway (Figure 3-10B panel d). In support of this observation, Bak and Bax deficient Jurkat cells were also completely resistant to VV811-

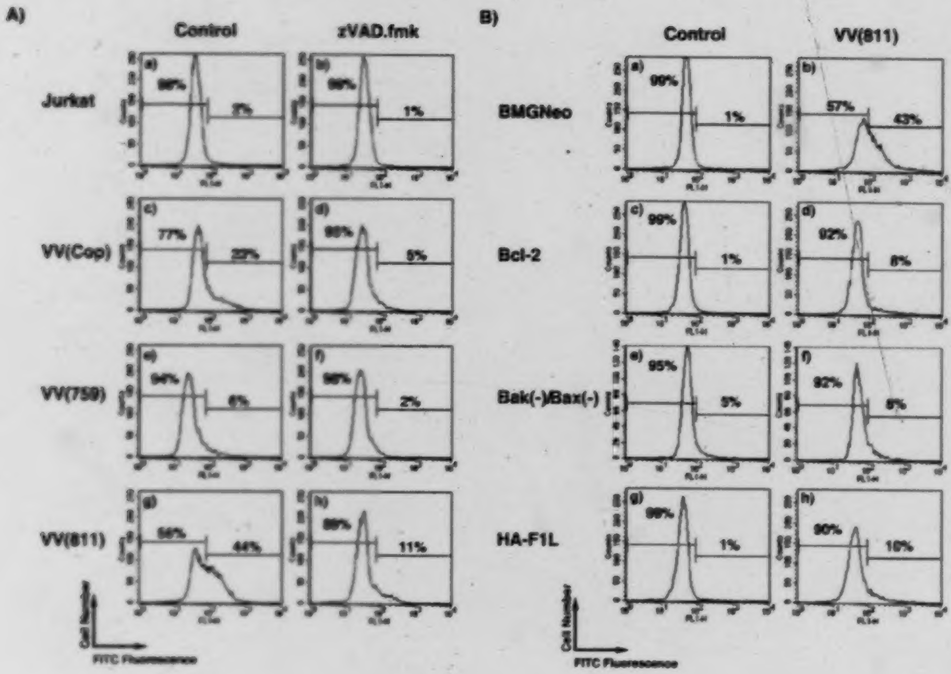


Figure 3-10 VV811 induces apoptosis that is inhibited by F1L.
Jurkat cells were mock infected or infected with either vaccinia strain Copenhagen, VV759 or VV811 at an MOI of 10. Fifteen hours post infection DNA fragmentation was monitored by TUNEL assay. (A) VV811 induces DNA fragmentation. (a) uninfected Jurkat cells; (b) uninfected Jurkat cells treated with 100 μM zVAD.fmk; (c) Jurkat cells infected with vaccinia virus strain Copenhagen; (d) Jurkat cells infected with vaccinia virus strain Copenhagen in the presence of 100μm zVAD.fmk; (e) Jurkat cells infected with VV759; (f) Jurkat cells infected with VV759 in the presence of 100 μM zVAD.fmk; (g) Jurkat cells infected with VV811; (h) Jurkat cells infected with VV811 in the presence of 100μm zVAD.fmk. (B) VV811-induced DNA fragmentation is inhibited by F1L. (a) Jurkat BMGneo cells; (b) Jurkat BMGneo cells infected with VV811; (c) Jurkat cells overexpressing Bcl-2; (d) Jurkat cells overexpressing Bcl-2 infected with VV811; (e) Bak and Bax deficient Jurkat cells; (f) Bak and Bax deficient Jurkat cells infected with VV811; (g) Jurkat cells stably expressing F1L; (h) Jurkat cells stably expressing F1L infected with VV811.

induced apoptosis (Figure 3-10A panel f). We also found that VV811-induced apoptosis was completely inhibited when F1L expressing Jurkat cells were infected. This observation clearly indicated that the presence of F1L could block VV811-induced apoptosis and further suggests that F1L expression during virus infection is essential for blocking the intrinsic apoptotic pathway that is inadvertently triggered by vaccinia virus infection.

3.4 Discussion

Successful virus infection requires that viruses counteract numerous anti-viral responses inflicted by the host immune system. The Poxviridae family, of which vaccinia is a member, encode proteins aimed at interfering with the host immune response and apoptosis (19, 50). The best-studied anti-apoptotic protein encoded by members of the poxvirus family is the caspase 8 inhibitor CrmA (57, 70). However, caspase 8-independent pathways occur, suggesting that inhibition of caspase 8 may not be sufficient to inhibit all apoptotic triggers. The important role of mitochondria in the apoptotic cascade led us to hypothesize that vaccinia virus would additionally encode a protein to block the mitochondrial component of apoptosis. Although the genome of vaccinia virus strain Copenhagen was completely sequenced in 1990, sequence analysis failed to reveal the presence of any obvious anti-apoptotic proteins that regulate the mitochondrial component suggesting that vaccinia virus might encode a novel inhibitor (21). In support of this we previously demonstrated that cells infected with vaccinia virus strain Copenhagen, which is naturally devoid of the caspase inhibitor CrmA, still displayed resistance to apoptosis and that vaccinia virus strain Copenhagen inhibited release of cytochrome c from the mitochondria (65). We now report that the vaccinia virus protein F1L is responsible for inhibiting the mitochondrial arm of the apoptotic response.

Since our previous data clearly demonstrated that vaccinia virus employed an unknown mechanism to inhibit apoptosis, we set out to identify the vaccinia virus gene(s) required. To achieve this goal we utilized two vaccinia virus deletion mutants, VV811 and VV759 (45). VV759 is missing 18 open reading frames while VV811 is missing 55 open reading frames. Our data demonstrated that although VV759 was completely competent for apoptosis inhibition, VV811 was not, indicating that genes essential for apoptosis inhibition were missing from VV811. To determine which open reading frame(s) missing from VV811 was necessary for blocking apoptosis we employed an infection and transfection strategy where cells were infected with VV811 and individual genes, placed under the control of a poxvirus promoter, were transfected into VV811 infected cells. Using this strategy we found that expression of the F1L open reading frame could restore apoptosis inhibition during VV811 infection.

Evidence now clearly indicates that mitochondria are central regulators of the apoptotic response by serving to directly initiate apoptosis in some situations and serving an amplification role in other situations (14, 33, 41, 64). Apoptosis results in both structural and physiological alterations to the mitochondria which include loss of the inner mitochondrial membrane potential, and the release of pro-apoptotic proteins. Mitochondrial released cytochrome c plays an essential role in apoptosis by promoting the activation of caspase 9 and members of the Bcl-2 family, both pro- and anti-apoptotic, regulate the release of cytochrome c (36). We found that the expression of F1L inhibited loss of the inner membrane potential and post-mitochondrial events by blocking the release of cytochrome c. Additionally, F1L expression also blocked release of SMAC from the mitochondria which functions by blocking the caspase-inhibitory action of cellular IAPs thereby augmenting the apoptotic cascade (15). By encoding F1L, vaccinia virus blocks the release of pro-apoptotic proteins, including cytochrome c release, the pivotal commitment step in the cascade, subsequently interfering with stimuli that initiate apoptosis at mitochondria and inhibiting the mitochondrial amplification loop.

Our confocal studies revealed that F1L localized to the mitochondria during infection as well as in the absence of infection and localization to the mitochondria requires the presence of a C-terminal domain. This domain encompasses a transmembrane region flanked by positive charges and a short hydrophilic tail. By appending the C-terminal domain of F1L onto EGFP we found that this domain was sufficient for mitochondrial localization. As such, F1L is a new member of a growing class of tail-anchored proteins that are specifically targeted to cellular membranes (66). Included in this class are proteins that target to the outer mitochondrial membrane, including Bcl-2 (3). However, unlike Bcl-2, which localizes to the ER and nuclear membrane in addition to the mitochondria, we find that F1L localizes predominantly to the mitochondria where it functions to inhibit apoptosis.

In order to inhibit the mitochondrial component of the apoptotic cascade, a large number of viruses encode obvious Bcl-2 homologues (13). Members of the Bcl-2 family contain up to four different Bcl-2 homology (BH) domains that are important for function (24). Data base searches have revealed no cellular proteins with homology to F1L. In particular we were unable to detect any homology to members of the Bcl-2 family of mitochondrial regulators including homology to the BH domains. Homology was only detected within the conserved C-terminal localization domain. This lack of homology indicates that F1L is a novel virus-encoded mitochondrial regulator. Recently, two other novel proteins that function to inhibit apoptosis at the mitochondria have been identified in HCMV and in the rabbit specific poxvirus, myxoma virus (17, 18, 22). vMIA, from HCMV, and M11L, from myxoma virus, both localize to mitochondria and block the release of cytochrome c (17, 18, 22). F1L is now the third member of this class of novel viral proteins that localize to the mitochondria and inhibits apoptosis by blocking release of cytochrome c.

F1L orthologs are present only in members of the Orthopoxvirus genus while members of the *Leporipoxvirus*, *Capripoxvirus*, *Yatapoxvirus* and *Suipoxvirus* genera contain M11L orthologs. At present the only poxvirus known to contain a Bcl-2 homologue is fowlpox virus which was identified based upon the presence of conserved BH domains (2, 13). All the F1L orthologs sequenced to date display greater than 90% sequence identity within the final 226 amino acids. The region of greatest diversity occurs at the N-terminus where strains of variola virus and ectromelia virus contain unique repeats. The significance of these repeats is currently unknown but cells infected with ectromelia virus are protected from apoptosis at the level of the mitochondria suggesting that the F1L ortholog is functional (J. Taylor and M. Barry and unpublished data).

The importance of the mitochondria during apoptosis and the presence of either F1L, M11L or fowlpox Bcl-2 in all poxviruses sequenced to date suggests that regulation of the mitochondrial apoptotic response is important for this family of viruses. Our studies demonstrate that F1L expression is capable of inhibiting both extrinsic apoptotic stimuli, in the form of anti-Fas and staurosporine, and intrinsic stimuli. In support of this, we found that the deletion virus VV811, which is devoid of F1L, stimulated an apoptotic response in Jurkat cells. A similar apoptotic response was observed in RL-5 cells infected with a mutant myxoma virus missing M11L (39). In our studies we observed that VV811-induced apoptosis occurred via an intrinsic mitochondrial route which was inhibited by overexpressing Bcl-2 and in cells deficient in the pro-apoptotic Bcl-2 family member Bak. Additionally, the expression of F1L was also capable of inhibiting apoptosis induced by VV811 infection by inhibiting cytochrome c release. These observations suggest that the presence of F1L is essential for inhibiting the intrinsic cellular suicide response initiated inadvertently during infection.

The exact mechanism of cytochrome c release and its regulation by Bcl-2 family members is currently undefined and controversial and the recent observation that cytochrome c release is associated with extensive remodeling of the inner mitochondrial membrane add another level of complexity (48). Three theories have been postulated to explain the release of cytochrome c (41, 46, 49). The first suggests that Bcl-2 family members form pores through which cytochrome c is released, while the second theory suggests that cytochrome c release occurs by global permeabilization of the mitochondrial membrane. The third theory suggests that cytochrome c release is controlled by a multiprotein complex known as the permeability transition pore (PTP). The PTP consists of the outer-mitochondrial-membrane localized voltage-dependent anion carrier, the inner-membrane localized adenine nucleotide translocator, the matrix protein cyclophilin D and two accessory proteins hexokinase and the peripheral benzodiazepine receptor (12, 60). Members of the Bcl-2 family associate with components of the pore leading to the suggestion that Bcl-2 family member function by regulating the pore (42, 51). Intriguingly, both vMIA and M11L interact with components of the permeability pore suggesting that regulation of the PT pore is important during infection (18, 22). F1L could potentially function by directly interacting with either members of the Bcl-2 family or by regulating the PT pore. In support of this, we previously observed that mitochondria purified from infected cells were resistant to cytochrome c release when treated with atractyolside, an adenine nucleotide translocator ligand, suggesting that vaccina virus regulates the PT pore (65). We are currently pursuing experiments to determine the exact mechanism of action of F1L.

The identification of F1L in the genome of vaccinia virus clearly indicates the importance of regulating the mitochondrial apoptotic response and continues to demonstrate that vaccinia virus is striving to outplay the host immune response. Members of the poxvirus family encode multiple anti-apoptotic proteins with distinct mechanisms of action (19). This is undoubtedly reflective of the extensive array of extrinsic and intrinsic apoptotic stimuli and the complexity of the apoptotic pathways and the wide range of cell types infected within the host. The existence of novel mitochondrial-localized proteins encoded by poxviruses, as well as the recently identified novel protein vMIA in HCMV, suggests that other pathogens may encode anti-apoptotic proteins unrelated to Bcl-2. The identification of F1L provides us with yet another hint of what viruses are capable of. Although the exact molecular mechanism of action of F1L remains to be determined, it is certain that F1L will be an essential tool for unraveling the complexities of the mitochondrial apoptotic pathway.

3.5 References

1. **Adams, J. M., and S. Cory.** 1998. The Bcl-2 protein family: arbiters of cell survival. Science **281:**1322-6.
2. **Afonso, C. L., E. R. Tulman, Z. Lu, L. Zsak, G. F. Kutish, and D. L. Rock.** 2000. The genome of fowlpox virus. J Virol **74:**3815-31.
3. **Akao, Y., Y. Otsuki, S. Kataoka, Y. Ito, and Y. Tsujimoto.** 1994. Multiple subcellular localization of bcl-2: detection in nuclear outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes. Cancer Res **54:**2468-71.
4. **Barry, M., and R. C. Bleackley.** 2002. Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol **2:**401-9.
5. **Barry, M., J. A. Heibein, M. J. Pinkoski, S. F. Lee, R. W. Moyer, D. R. Green, and R. C. Bleackley.** 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol **20:**3781-94.
6. **Barry, M., S. Hnatiuk, K. Mossman, S. F. Lee, L. Boshkov, and G. McFadden.** 1997. The myxoma virus M-T4 gene encodes a novel RDEL-containing protein that is retained within the endoplasmic reticulum and is important for the productive infection of lymphocytes. Virology **239:**360-77.
7. **Barry, M., and G. McFadden.** 1998. Apoptosis regulators from DNA viruses. Curr Opin Immunol **10:**422-30.
8. **Bertrand, R., E. Solary, P. O'Connor, K. W. Kohn, and Y. Pommier.** 1994. Induction of a common pathway of apoptosis by staurosporine. Exp Cell Res **211:**314-21.
9. **Bossy-Wetzel, E., and D. R. Green.** 2000. Assays for cytochrome c release from mitochondria during apoptosis. Methods Enzymol **322:**235-42.
10. **Brenner, C., H. Cadiou, H. L. Vieira, N. Zamzami, I. Marzo, Z. Xie, B. Leber, D. Andrews, H. Duclohier, J. C. Reed, and G. Kroemer.** 2000. Bcl-2 and Bax regulate the channel activity of the mitochondrial adenine nucleotide translocator. Oncogene **19:**329-36.
11. **Crompton, M.** 2000. Bax, Bid and the permeabilization of the mitochondrial outer membrane in apoptosis. Curr Opin Cell Biol **12:**414-9.
12. **Crompton, M.** 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem J **341:**233-49.
13. **Cuconati, A., and E. White.** 2002. Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. Genes Dev **16:**2465-78.
14. **Desagher, S., and J. C. Martinou.** 2000. Mitochondria as the central control point of apoptosis. Trends Cell Biol **10:**369-77.
15. **Du, C., M. Fang, Y. Li, L. Li, and X. Wang.** 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell **102:**33-42.
16. **Ehrenberg, B., V. Montana, M. D. Wei, J. P. Wuskell, and L. M. Loew.** 1988. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. Biophys J **53:**785-94.
17. **Everett, H., M. Barry, S. F. Lee, X. Sun, K. Graham, J. Stone, R. C. Bleackley, and G. McFadden.** 2000. M11L: a novel mitochondria-localized protein of myxoma virus that blocks apoptosis of infected leukocytes. J Exp Med **191:**1487-98.
18. **Everett, H., M. Barry, X. Sun, S. F. Lee, C. Frantz, L. G. Berthiaume, G. McFadden, and R. C. Bleackley.** 2002. The Myxoma Poxvirus Protein, M11L, Prevents Apoptosis by Direct Interaction with the Mitochondrial Permeability Transition Pore. J Exp Med **196:**1127-1140.
19. **Everett, H., and G. McFadden.** 2002. Poxviruses and apoptosis: a time to die. Curr Opin Microbiol **5:**395.
20. **Feng, P., J. Park, B. S. Lee, S. H. Lee, R. J. Bram, and J. U. Jung.** 2002. Kaposi's sarcoma-associated herpesvirus mitochondrial K7 protein targets a cellular calcium-modulating cyclophilin ligand to modulate intracellular calcium concentration and inhibit apoptosis. J Virol **76:**11491-504.
21. **Goebel, S. J., G. P. Johnson, M. E. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete DNA sequence of vaccinia virus. Virology **179:**247-66, 517-63.
22. **Goldmacher, V. S., L. M. Bartle, A. Skaletskaya, C. A. Dionne, N. L. Kedersha, C. A. Vater, J. W. Han, R. J. Lutz, S. Watanabe, E. D. Cahir McFarland, E. D. Kieff, E. S. Mocarski, and**

T. Chittenden. 1999. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc Natl Acad Sci U S A **96:**12536-41.

23. **Grosenbach, D. W., D. O. Ulaeto, and D. E. Hruby.** 1997. Palmitylation of the vaccinia virus 37-kDa major envelope antigen. Identification of a conserved acceptor motif and biological relevance. J Biol Chem **272:**1956-64.
24. **Gross, A., J. M. McDonnell, and S. J. Korsmeyer.** 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev **13:**1899-911.
25. **Gross, A., X. M. Yin, K. Wang, M. C. Wei, J. Jockel, C. Milliman, H. Erdjument-Bromage, P. Tempst, and S. J. Korsmeyer.** 1999. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem **274:**1156-63.
26. **Hengartner, M. O.** 2000. The biochemistry of apoptosis. Nature **407:**770-6.
27. **Hnatiuk, S., M. Barry, W. Zeng, L. Liu, A. Lucas, D. Percy, and G. McFadden.** 1999. Role of the C-terminal RDEL motif of the myxoma virus M-T4 protein in terms of apoptosis regulation and viral pathogenesis. Virology **263:**290-306.
28. **Janiak, F., B. Leber, and D. W. Andrews.** 1994. Assembly of Bcl-2 into microsomal and outer mitochondrial membranes. J Biol Chem **269:**9842-9.
29. **Jensen, O. N., T. Houthaeve, A. Shevchenko, S. Cudmore, T. Ashford, M. Mann, G. Griffiths, and J. Krijnse Locker.** 1996. Identification of the major membrane and core proteins of vaccinia virus by two-dimensional electrophoresis. J Virol **70:**7485-97.
30. **Johnston, J. B., and G. McFadden.** 2003. Poxvirus immunomodulatory strategies: current perspectives. J Virol **77:**6093-100.
31. **Karasuyama, H., and F. Melchers.** 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. Eur J Immunol **18:**97-104.
32. **Kettle, S., A. Alcami, A. Khanna, R. Ehret, C. Jassoy, and G. L. Smith.** 1997. Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1beta-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1beta-induced fever. J Gen Virol **78 (Pt 3):**677-85.
33. **Kroemer, G., and J. C. Reed.** 2000. Mitochondrial control of cell death. Nat Med **6:**513-9.
34. **Li, H., H. Zhu, C. J. Xu, and J. Yuan.** 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell **94:**491-501.
35. **Li, L. Y., X. Luo, and X. Wang.** 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature **412:**95-9.
36. **Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang.** 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell **91:**479-89.
37. **Liu, X., C. N. Kim, J. Yang, R. Jemmerson, and X. Wang.** 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86:**147-57.
38. **Luo, X., I. Budihardjo, H. Zou, C. Slaughter, and X. Wang.** 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell **94:**481-90.
39. **Macen, J. L., K. A. Graham, S. F. Lee, M. Schreiber, L. K. Boshkov, and G. McFadden.** 1996. Expression of the myxoma virus tumor necrosis factor receptor homologue and M11L genes is required to prevent virus-induced apoptosis in infected rabbit T lymphocytes. Virology **218:**232-7.
40. **Marchetti, P., T. Hirsch, N. Zamzami, M. Castedo, D. Decaudin, S. A. Susin, B. Masse, and G. Kroemer.** 1996. Mitochondrial permeability transition triggers lymphocyte apoptosis. J Immunol **157:**4830-6.
41. **Martinou, J. C., and D. R. Green.** 2001. Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol **2:**63-7.
42. **Marzo, I., C. Brenner, N. Zamzami, J. M. Jurgensmeier, S. A. Susin, H. L. Vieira, M. C. Prevost, Z. Xie, S. Matsuyama, J. C. Reed, and G. Kroemer.** 1998. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science **281:**2027-31.
43. **Metivier, D., B. Dallaporta, N. Zamzami, N. Larochette, S. A. Susin, I. Marzo, and G. Kroemer.** 1998. Cytofluorometric detection of mitochondrial alterations in early CD95/Fas/APO-

1-triggered apoptosis of Jurkat T lymphoma cells. Comparison of seven mitochondrion-specific fluorochromes. Immunol Lett **61**:157-63.

44. **Nguyen, M., D. G. Millar, V. W. Yong, S. J. Korsmeyer, and G. C. Shore.** 1993. Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. J Biol Chem **268**:25265-8.

45. **Perkus, M. E., S. J. Goebel, S. W. Davis, G. P. Johnson, E. K. Norton, and E. Paoletti.** 1991. Deletion of 55 open reading frames from the termini of vaccinia virus. Virology **180**:406-10.

46. **Reed, J. C., and D. R. Green.** 2002. Remodeling for demolition: changes in mitochondrial ultrastructure during apoptosis. Mol Cell **9**:1-3.

47. **Roulston, A., R. C. Marcellus, and P. E. Branton.** 1999. Viruses and apoptosis. Annu Rev Microbiol **53**:577-628.

48. **Scorrano, L., M. Ashiya, K. Buttle, S. Weiler, S. A. Oakes, C. A. Mannella, and S. J. Korsmeyer.** 2002. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell **2**:55-67.

49. **Scorrano, L., and S. J. Korsmeyer.** 2003. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun **304**:437-44.

50. **Seet, B. T., J. B. Johnston, C. R. Brunetti, J. W. Barrett, H. Everett, C. Cameron, J. Sypula, S. H. Nazarian, A. Lucas, and G. McFadden.** 2003. Poxviruses and immune evasion. Annu Rev Immunol **21**:377-423.

51. **Shimizu, S., A. Konishi, T. Kodama, and Y. Tsujimoto.** 2000. BH4 domain of antiapoptotic Bcl-2 family members closes voltage- dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. Proc Natl Acad Sci U S A **97**:3100-5.

52. **Smith, G. L., and G. McFadden.** 2002. Smallpox: anything to declare? Nat Rev Immunol **2**:521-7.

53. **Stuart, D., K. Graham, M. Schreiber, C. Macaulay, and G. McFadden.** 1991. The target DNA sequence for resolution of poxvirus replicative intermediates is an active late promoter. J Virol **65**:61-70.

54. **Susin, S. A., H. K. Lorenzo, N. Zamzami, I. Marzo, B. E. Snow, G. M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D. R. Goodlett, R. Aebersold, D. P. Siderovski, J. M. Penninger, and G. Kroemer.** 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature **397**:441-6.

55. **Suzuki, Y., Y. Imai, H. Nakayama, K. Takahashi, K. Takio, and R. Takahashi.** 2001. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell **8**:613-21.

56. **Tafani, M., D. A. Minchenko, A. Serroni, and J. L. Farber.** 2001. Induction of the mitochondrial permeability transition mediates the killing of HeLa cells by staurosporine. Cancer Res **61**:2459-66.

57. **Tewari, M., and V. M. Dixit.** 1995. Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus crmA gene product. J Biol Chem **270**:3255-60.

58. **Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh.** 2000. Viral subversion of the immune system. Annu Rev Immunol **18**:861-926.

59. **Tschopp, J., M. Thome, K. Hofmann, and E. Meinl.** 1998. The fight of viruses against apoptosis. Curr Opin Genet Dev **8**:82-7.

60. **Verrier, F., B. Mignotte, G. Jan, and C. Brenner.** 2003. Study of PTPC composition during apoptosis for identification of viral protein target. Ann N Y Acad Sci **1010**:126-42.

61. **Vieira, H. L., A. S. Belzacq, D. Haouzi, F. Bernassola, I. Cohen, E. Jacotot, K. F. Ferri, C. El Hamel, L. M. Bartle, G. Melino, C. Brenner, V. Goldmacher, and G. Kroemer.** 2001. The adenine nucleotide translocator: a target of nitric oxide, peroxynitrite, and 4-hydroxynonenal. Oncogene **20**:4305-16.

62. **Wang, G. Q., E. Wieckowski, L. A. Goldstein, B. R. Gastman, A. Rabinovitz, A. Gambotto, S. Li, B. Fang, X. M. Yin, and H. Rabinowich.** 2001. Resistance to granzyme B-mediated cytochrome c release in Bak-deficient cells. J Exp Med **194**:1325-37.

63. **Wang, H. W., T. V. Sharp, A. Koumi, G. Koentges, and C. Boshoff.** 2002. Characterization of an anti-apoptotic glycoprotein encoded by Kaposi's sarcoma-associated herpesvirus which resembles a spliced variant of human survivin. Embo J **21**:2602-15.

64. **Wang, X.** 2001. The expanding role of mitochondria in apoptosis. Genes Dev **15**:2922-33.

65. **Wasilenko, S. T., A. F. Meyers, K. Vander Helm, and M. Barry.** 2001. Vaccinia virus infection disarms the mitochondrion-mediated pathway of the apoptotic cascade by modulating the permeability transition pore. J Virol **75:**11437-48.

66. **Wattenberg, B., and T. Lithgow.** 2001. Targeting of C-terminal (tail)-anchored proteins: understanding how cytoplasmic activities are anchored to intracellular membranes. Traffic **2:**66-71.

67. **Whitaker, J. E., P. L. Moore, and R. P. Haugland.** 1991. Dihydrotetramethylrosamine: a long wavelength, fluorogenic peroxidase substrate evaluated in vitro and in a model phagocyte. Biochem Biophys Res Commun **175:**387-93.

68. **Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J. L. Vayssiere, P. X. Petit, and G. Kroemer.** 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med **181:**1661-72.

69. **Zha, J., H. Harada, E. Yang, J. Jockel, and S. J. Korsmeyer.** 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell **87:**619-28.

70. **Zhou, Q., S. Snipas, K. Orth, M. Muzio, V. M. Dixit, and G. S. Salvesen.** 1997. Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J Biol Chem **272:**7797-800.

Chapter 4: The Vaccinia Virus F1L Protein Interacts with the Proapoptotic Protein Bak and Inhibits Bak Activation

Preface

The data presented in all figures was generated by myself with the exception of Figure 4-1C which was generated by Dave Bond and Figure 4-5B which was generated by Logan Banadyga. I wrote the first draft of the manuscript. A major editorial contribution from my supervisor, Dr. Michele Barry, led to the final version of the paper. A version of this chapter has been accepted for publication in the Journal of Virology.

4.1 Introduction

Apoptosis is an evolutionary conserved pathway that is important for development, cellular homeostasis and protection from microbial pathogens (38). Although the central tenets of the apoptotic process involve the activation of a family of cysteine proteases, referred to as caspases, mitochondria act as a critical control point during apoptosis (22, 55). Following an apoptotic insult, mitochondria undergo loss of the inner mitochondrial membrane potential and release of an array of death promoting proteins including apoptosis inducing factor (AIF), endonuclease G, SMAC/Diablo, HtrA2/Omi, and cytochrome c (22, 55).

The mitochondrial component of the apoptotic cascade is tightly regulated by members of the Bcl-2 family (10, 25, 44). The Bcl-2 family consists of both anti- and pro-apoptotic members that function to either maintain mitochondrial integrity or stimulate the release of cytochrome c (10, 25, 44). Bcl-2 family members are typically characterized as containing one or more Bcl-2 homology (BH) domains (10, 25). Bak and Bax, two pro-apoptotic members of the Bcl-2 family, are activated in response to apoptotic stimuli and play a pivotal role in generating apoptotic death (34, 59). In the absence of an apoptotic trigger, Bax is predominantly cytoplasmic or loosely associated with intracellular membranes (60). Following an apoptotic trigger Bax undergoes a series of conformational changes which include exposure of the N-terminus and liberation of the C-terminal transmembrane domain resulting in mitochondrial membrane insertion, followed by subsequent homo-oligomerization and release of cytochrome c (2, 7). In contrast to Bax, the majority of Bak normally resides at the mitochondria. Similar to Bax activation, apoptosis triggers a multi-step activation of Bak resulting in a conformational change in Bak and subsequent homo-oligomerization (24, 58). Anti-apoptotic members of the Bcl-2 family, such as Bcl-XL and Bcl-2, prevent Bax and Bak oligomerization thereby interfering with cyctochrome c release (25, 44). Cells deficient in Bax and Bak are protected from apoptosis initiated by a wide range of stimuli clearly establishing the importance of Bak and Bax in apoptotic cells (34, 59).

To inhibit apoptosis many viruses encode proteins that function directly at the mitochondrial checkpoint (5, 6). For example, a number of viruses encode obvious Bcl-2 homologues that function at the mitochondria to inhibit the release of cytochrome c and apoptosis (13, 26, 40). More recently however, novel viral proteins that lack homology to Bcl-2 have been described that also function to inhibit the release of cytochrome c (5, 6). These include K7, encoded by Kaposi's sarcoma associated herpesvirus, vMIA (viral mitochondria-localized inhibitor of apoptosis) encoded by human cytomegalovirus (HCMV) and M11L encoded by myxoma virus (16, 21, 54). K7 is related to a spliced version of human survivin but also contains a portion of a baculovirus inhibitor of apoptosis repeat (BIR) domain and a putative BH2 region (54). K7 localizes to mitochondria and interacts with activated caspase 3, Bcl-2 and calcium-modulating cyclopilin ligand (CAML) resulting in the regulation of cellular calcium levels and effectively protecting cells from apoptosis (19, 54). VMIA encoded by HCMV inhibits release of cytochrome c by a unique mechanism (21). The expression of vMIA causes the constitutive mitochondrial localization of Bax and the formation of Bax oligomers, however, despite demonstrating Bax oligomerization, vMIA effectively inhibits cytochrome c release (3, 41). M11L, encoded by the poxvirus myxoma virus, localizes to mitochondria and inhibits the release of cytochrome c (16, 17). M11L constitutively interacts with Bak and the peripheral benzodiazepine receptor (PBR), a component of the permeability transition pore, which may be involved in cytochrome c release (17, 29, 52). M11L homologues exist in a subset of poxviruses including members of the Leporipoxvirus, Suipoxvirus, Capripoxvirus and Yatapoxvirus genera. At present the only poxviruses known to contain obvious Bcl-2 homologues are fowlpox virus and canary poxvirus, two members of the Avipoxvirus genus (1, 51). The FPV039 open reading frame in fowlpox virus was initially identified based upon the presence of obvious BH1 and BH2 domains and is predicted to localize to the mitochondria and inhibit cytochrome c release (1).

We recently identified F1L as an anti-apoptotic protein expressed during vaccinia virus infection (47, 57). F1L lacks obvious sequence homology to M11L as well as cellular members of the Bcl-2 family. F1L localizes to the mitochondria and inhibits apoptosis by blocking the release of cytochrome c and preventing the loss of the inner mitochondrial membrane potential by an unknown mechanism (47, 57). We now report that F1L interacts with the pro-apoptotic protein Bak. F1L expression inhibits both Bak oligomerization and the initial N-terminal exposure of Bak, two necessary features for Bak pro-apoptotic function. Notably, a recombinant vaccinia virus deleted for F1L induces apoptosis upon infection, indicating that the presence of F1L is necessary to inhibit apoptosis initiated by virus infection.

4.2 Materials and Methods

Cells and Viruses.

Jurkat and HeLa cells were cultured as previously described (47, 57). Bak and Bax deficient Jurkat cells were a gift from H. Rabinowich, (University of Pittsburgh School of Medicine, Pittsburgh, PA) (53). CV-1 and HEK 293T cells were grown in DMEM (Gibco Invitrogen Inc.) supplemented with 10% fetal bovine serum (FBS) (Gibco Invitrogen Inc.), 2mM L-glutamine, 50U/ml penicillin, and 50µg/ml streptomycin. Murine embryonic fibroblasts (MEFs), were provided by S. Korsmeyer, (Harvard Medical School, Boston, MA) and cultured as described (59). Vaccinia virus strain Copenhagen (VV(Cop)) and VV(Cop)-EGFP were provided by G. McFadden (Robarts Research Institute, London, Ontario). Vaccinia virus strain Western Reserve (WR) expressing a Flag-tagged F1L (VV(WR)Flag-F1L) was generated as described (47). Viruses were routinely grown in Baby Green Monkey Kidney (BGMK) cells. Single step growth curves were generated by infecting CV-1 cells at an MOI of 2 for 1 hour. Infected cells were harvested at 0, 4, 8, 12 and 24 hours post infection and viral titers determined by plaque formation on CV-1 cells.

Generation of F1L Deletion Virus.

F1L gene fragments corresponding to the first 180 nucleotides and the last 166 nucleotides were amplified by polymerase chain reaction (PCR) using the following primer pairs: F1L(F) (5'-CTCGAGATGTTGTCGATGTTTATG-3') and F1L 2.2 (5'-AATGCAGATCTGGATCTGATAGATAATCGAGTATGT-3)', F1L(R) (5'-GGATCCT TATCCTATCATGTATTT–3') and F1L2.3 (5'-GATCCAGATCTGCATTCTATCGCA

TACTATCGCATACTATATGCGA–3'). The resulting amplified gene fragments contained a complementary linker with a BglII restriction site. The F1L gene fragments served as templates for overlapping PCR with primers F1L(F) and F1L(R) to generate an F1L fragment, F1Lf/b, containing a BglII restriction site which was subsequently cloned into pGEMT (Promega) to generate pGEMT-F1Lf/b. EGFP under the control of an early/late poxvirus promoter was amplified by PCR from pSC66-EGFP using primers E/L-Syn(BglII) (5'-AGATCTAAAAATTGAAATTTTATTTT-3') and EGFPR(BglII) (5'-AGATCTTTACTTGTACAGCTCGTCCATGCC-3'). The resulting EGFP gene fragment was subcloned into pGEMT-F1Lf/b via the internal BglII restriction site to generate pGEMT-F1Lf/b-EGFP. VV(Cop) was used to generate VV(Cop)ΔF1L by homologous recombination as previously described (14). BGMK cells (1×10^6) were infected with VV(Cop) at an MOI of 0.05 and transfected with pGEMT-F1Lf/b-EGFP using Lipofectin (Invitrogen Life Technologies). Recombinant viruses were selected by EGFP fluorescence, plaque purified and the presence of the disrupted F1L open reading frame (ORF) was confirmed by PCR.

Gel Filtration Analysis.

Jurkat cells (1×10^7) were lysed in CHAPS lysis buffer containing 2% CHAPS w/v (Sigma Chemical Co.), 300mM NaCl, 20mM Tris pH 7.4, 2mM EDTA and 0.2mM DTT. Cell lysates were centrifuged at 16,000×g for 15 minutes and the soluble fractions retained. Gel filtration chromatography was performed at 4°C using a Superose6 HR (10/30) column (GE Healthcare) equilibrated with 1% CHAPS lysis buffer. The Superose6 column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), and ovalbumin (43 kDa) (GE Healthcare). Cell lysates (1.3mg) were applied to the column and eluted at a flow rate of 0.150ml/minute. Fractions of 150μl were collected and analyzed by western blot for the presence of Bak.

Immunoaffinity Purification.

Immunoaffinity experiments were performed on a HiTrap NHS-activated HP affinity column (GE Healthcare) coupled with 9.6mg of protein G purified rabbit anti-F1L antibody according to manufacturers' protocol (GE Healthcare) (47). HeLa cells (1×10^7) were either mock infected or infected with VV(WR)Flag-F1L, and lysed in CHAPS lysis buffer containing 2% CHAPS w/v, 137mM NaCl, and 20mM Tris pH 7.4. Cell lysates were applied to the anti-F1L affinity column and washed with 2% CHAPS lysis buffer. Bound proteins were eluted over a total volume of 5ml using a linear gradient with elution buffer containing 100mM glycine, 0.5M NaCl pH2.7. One ml elution fractions were collected and analyzed by western blot for the presence of F1L-Flag, Bak, Bax, Bcl-2, Bcl-XL, and Mcl-1.

Immunoprecipitation.

HEK 293T (4×10^6) cells were transfected with 4μg of pcDNA3-HA-Bak and pEGFP-F1L using Lipofectamine 2000 (GIBCO BRL Technologies Inc.). Cells were lysed in 2% CHAPS w/v, 150mM NaCl, 50mM Tris pH8.0 containing protease inhibitors (Roche). Cell lysates were immunoprecipitated with either goat anti-EGFP antibody (Dr. Luc Berthiaume, University of Alberta, Canada) or mouse anti-HA (12CA5). To detect interaction during virus infection, HeLa cells (8×10^6) were transfected with 14μg of either pSC66 or pSC66-F1L-Flag and infected with VV(Cop) at a MOI of 10. Whole cell lysates were prepared in CHAPS lysis buffer containing 2% CHAPS w/v, 137mM NaCl, and 20mM Tris pH 7.4, 20mM EDTA containing 10% glycerol and complexes precipitated using magnetic beads (Dynabeads M-280 Tosylactivated) (Dynal Biotech.) coated with either anti-Bak NT (Upstate) or anti-Flag M2 (Sigma Aldrich) as specified by the manufacturer. To detect F1L interaction with endogenous Bak in the absence of infection, 8×10^6 HeLa cells were transfected with 14μg of either pEGFP-C3 (Clontech) or pEGFP-F1L and immunoprecipitated with goat anti-EGFP antibody.

Mitochondria Purification and Cytochrome c Release.

Cellular fractionation into cytosolic and membranous/mitochondrial fractions was performed using digitonin as described (56). Cytochrome c release was monitored by western blotting both the supernatant and membranous fractions. Mitochondria were purified from MEFs as described (61). Briefly, MEFs were resuspended in hypotonic lysis buffer containing 250mM sucrose, 20mM HEPES pH 7.5, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA and 1mM EGTA and incubated on ice for 30 minutes with intermittent mixing. Cells were disrupted by passage through a 23 gauge needle and the lysates were centrifuged at 750×g for 10 minutes at 4°C. Mitochondria were isolated at 10,000×g for 20 minutes at 4°C and resuspended in 250mM sucrose, 20mM HEPES pH 7.5, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA and 150mM NaCl. Protein concentrations were determined by Bicinchoninic Acid (BCA) assay (Pierce Chemical Co.). To monitor cytochrome c release, 30μg of purified mitochondria were incubated with 5, 10, or 15ng caspase-8-cleaved recombinant human Bid (tBid) (R&D Systems Inc.) for 35 minutes at 30°C. Samples were centrifuged at 10,000×g for 15 minutes at 4°C to separate the mitochondrial pellet from the supernatant prior to SDS-PAGE analysis. Isolated mitochondria (30μg) from MEFs treated with tBid were subjected to cross-linking using 900μM of bismaleimidohexane (BMH) for 30 minutes (Pierce Chemical Co.). Crosslinking was terminated by the addition of SDS sample buffer containing 1mM DTT. For limited trypsin proteolysis 30μg of mitochondria were treated with tBid were resuspended in buffer containing 250mM sucrose, 20mM HEPES pH 7.5, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, and 150mM NaCl supplemented with 100μg/ml trypsin (Sigma Chemical Co.). Following 20 minutes on ice, mitochondria were collected by centrifugation at 10,000×g for 15 minutes at 4°C and resuspended in SDS sample buffer.

Confocal Microscopy.

The localization of Bak and F1L during virus infection was assessed by confocal microscopy. HeLa cells (5×10^5) seeded on 1mm glass coverslips were infected with VV(WR)Flag-F1L at an MOI of 5. Following an 8 hour infection, cells were fixed at room temperature for 30 minutes with 3% paraformaldehyde in PBS and permeabilized with 0.25% (w/v) Saponin (Sigma Chemical Co.). Cells were stained with 4 μg/mL rabbit anti-Bak antibody (G23) (Santa Cruz Biotechnology) and 10 μg/mL Alex Fluor 546 goat anti-rabbit antibody (Invitrogen Life Technologies). To detect Flag-F1L, cells were treated with 10 μg/mL anti-Flag M2 monoclonal antibody directly conjugated to

FITC (Sigma-Aldrich Inc.). Images were obtained using a LSM510 scanning microscope equipped with a 43 × 1.4 oil emersion Plan-Apochromat objective.

Bak Conformational Analysis by Flow Cytometry.

The conformational status of Bak was assessed as previously described (23, 24). Jurkat cells (1×10^6) were treated with 0.5μM staurosporine for 2 hours, fixed with 0.25% paraformaldehyde (Sigma Chemical Co.) and stained with 2μg/ml anti-Bak antibody (clone TC100) (Oncogene Research Products) or an antibody specific for NK1.1 (PK136) as an isotype control (28). Cells were counter stained with phycoerythrin (PE)-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc.). Antibody staining was detected through the FL-2 channel equipped with a 585 filter (42nm band pass). Data was acquired with fluorescence signals at logarithmic gain and was analyzed with CellQuest software.

Measurement of Mitochondrial Membrane Potential.

Changes in mitochondrial membrane potential were quantified in infected cells by staining cells with tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) as previously described (36, 57). Cells were loaded with TMRE by incubating cells in RPMI 1640 media containing 0.2 μM TMRE for 30 minutes at 37°C. Cells were also treated with a membrane uncoupler, carbonyl cyanide *m*-chlorophenyl hydrazone (ClCCP) (Sigma Chemical Co.) as described (57). TMRE fluorescence was acquired through the FL-2 channel equipped with a 585 filter (42nm band pass). Data was acquired with fluorescence signals at logarithmic gain. Data was analyzed with CellQuest software.

Immunoblotting.

Cell lysates were subjected to SDS PAGE analysis and transferred to PVDF membrane (GE Healthcare) and the following antibodies were used for detection: anti-cytochrome c (clone 7H8.2C12) (Pharmingen), polyclonal rabbit anti-caspase 3 (56), rabbit anti-EGFP (Dr. Luc Berthiaume, Edmonton, Alberta, Canada), anti-Flag M2-HRP conjugated antibody (Sigma Aldrich), anti-manganese superoxide dismutase (MnSOD) (SOD-111) (Stressgen Bioreagents), anti-Bak NT (Upstate), anti-Bax N20-HRP (Santa Cruz Biotechnology), anti-Bcl-2 (clone 124) (Upstate), anti-Bcl-XL (Santa Cruz Biotechnology), and anti-Mcl-1(clone RC22) (Lab Vision Corp.). Proteins were visualized by chemiluminescence according to the manufacturer's directions (GE Health Care).

4.3 Results

F1L Expression Protects Cells From Apoptosis.

To further understand the anti-apoptotic mechanism of F1L and its importance in modulating the apoptotic response during virus infection, we generated a F1L deletion virus. Using homologous recombination, the F1L ORF was disrupted by insertion of EGFP to create VV(Cop)ΔF1L (14). The recombinant VV(Cop)ΔF1L virus was plaque purified on CV-1 cells and PCR analysis indicated that the resulting VV(Cop)ΔF1L virus was pure (Figure 4-1A). No obvious difference in plaque morphology or growth in a single step growth curve were detected between the wildtype VV(Cop) and VV(Cop)ΔF1L virus following infection (Figure 4-1B and C) suggesting that F1L is dispensable for efficient virus replication during infection of CV-1 cells.

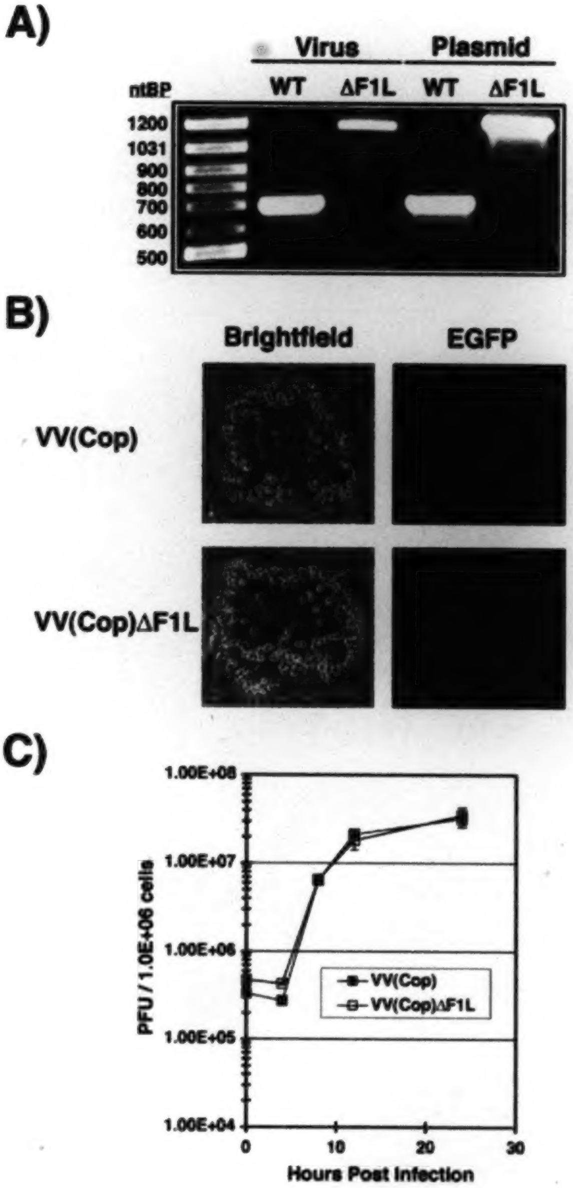


Figure 4-1 Characterization of a recombinant VV devoid of F1L
(A) Agarose gel analysis of PCR products amplified from VV(Cop) and VV(Cop)ΔF1L infected cells compared to plasmid containing wildtype F1L and the plasmid used to generate VV(Cop)ΔF1L by homologous recombination. 'WT' indicates wildtype F1L open reading frame; 'ΔF1L' indicates the F1L open reading frame disrupted by insertion of the EGFP gene (B) Microscopic analysis of plaques generated from BGMK cells infected with VV(Cop) and VV(Cop)ΔF1L. (C) Single step growth analysis of VV(Cop) and VV(Cop)ΔF1L in CV-1 cells.

To investigate the anti-apoptotic contribution of F1L during virus infection, Jurkat cells were infected with either VV(Cop) or VV(Cop)ΔF1L and five hours post-infection, apoptosis was triggered by treating cells with staurosporine. Apoptosis was measured by quantifying loss of the inner mitochondrial membrane potential by TMRE fluorescence, a hydrophobic cationic dye that is readily taken up by healthy respiring mitochondria (36). In the absence of staurosporine, all cell populations demonstrated high levels of TMRE fluorescence indicative of healthy non-apoptotic cells (Figure 4-2A, panel a, d, f, and h). Jurkat cells treated with the uncoupler ClCCP showed a clear loss of the inner membrane potential (Figure 4-2A, panel c) and treatment with staurosporine resulted in 50% of mock infected cell losing their inner mitochondrial membrane potential (Figure 4-2A, panel b). Jurkat cells overexpressing Bcl-2 and cells infected with VV(Cop) showed clear inhibition of the loss of the inner mitochondrial membrane potential (Figure 4-2A, panel e and g), while Jurkat cells infected with VV(Cop)ΔF1L no longer maintained the inner mitochondrial membrane potential indicating that in the absence of F1L, vaccinia virus was unable to inhibit staurosporine-induced apoptosis (Figure 4-2A, panel i).

Since infection with VV(Cop)ΔF1L was unable to inhibit loss of the inner mitochondrial membrane potential, we assessed the ability of VV(Cop)ΔF1L to inhibit both cytochrome c release and caspase 3 activation after treatment with staurosporine. To determine if VV(Cop)ΔF1L was also unable to inhibit the release of cytochrome c, Jurkat cells were infected, treated with staurosporine and fractionated into mitochondrial and cytosolic fractions and cytochrome c release monitored by western blotting. Mock infected cells treated with staurosporine displayed a loss of cytochrome c from the

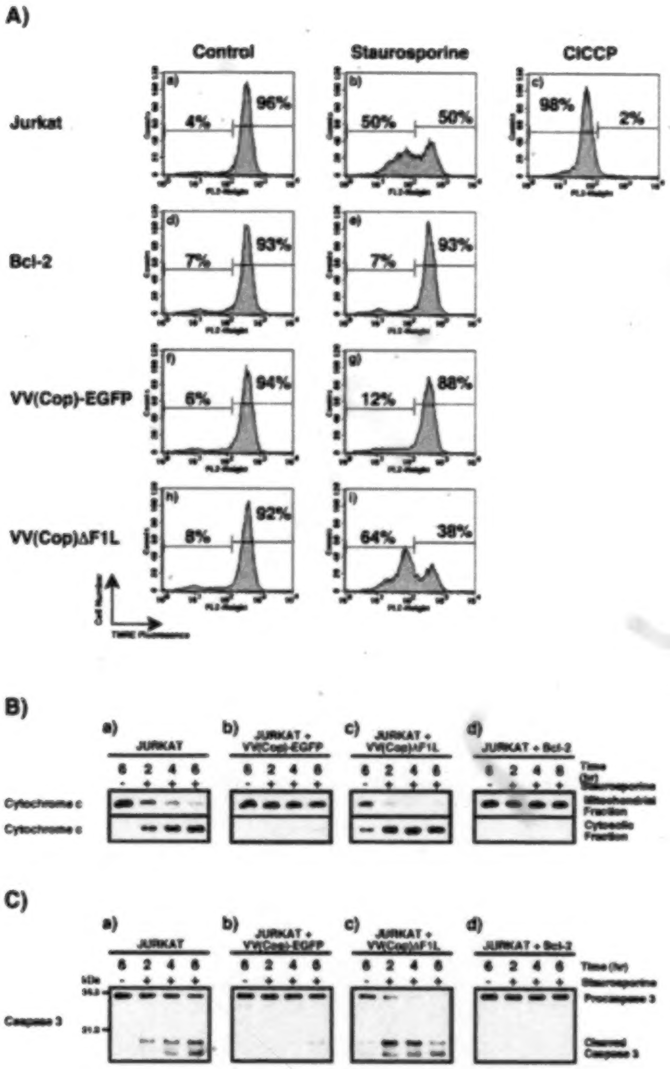


Figure 4-2 VV(Cop)ΔF1L is unable to protect cells from staurosporine-induced apoptosis.

(A) Jurkat and Jurkat cells overexpressing Bcl-2 were mock infected or infected with VV(Cop) or VV(Cop)ΔF1L at an MOI of 10 and treated with 500nM staurosporine for for 90 minutes to induce apoptosis. Apoptosis was assessed by TMRE fluorescence which measures loss of the inner mitochondrial membrane potential. (B) Jurkat cells and Jurkat cells overexpressing Bcl-2 were either mock infected, infected with VV(Cop) or infected with VV(Cop)ΔF1L and treated with 500nM staurosporine. Cytochrome c release was monitored by western blot analysis. (C) Jurkat cells and Jurkat cells overexpressing Bcl-2 were either mock infected, infected with VV(Cop) or infected with VV(Cop)ΔF1L at an MOI of 10 and treated 500nM with staurosporine. Caspase 3 activation was monitored by western blot analysis.

mitochondrial fraction to the cytosolic fraction (Figure 4-2B, panel a) which was completely blocked by overexpression of Bcl-2 and significantly impaired when cells were infected with VV(Cop) (Figure 4-2B, panel b and d). Cells infected with VV(Cop)ΔF1L however, were no longer able to inhibit cytochrome c release (Figure 4-2B, panel c). Similarly, cells infected with VV(Cop)ΔF1L and treated with staurosporine were no longer able to inhibit the activation of caspase 3 (Figure 4-2C, panel c). These results indicated that cells infected with VV(Cop)ΔF1L were incapable of inhibiting the mitochondrial dependent apoptotic cascade including the loss of cytochrome c and the loss of the inner membrane potential. Even in the absence of staurosporine, infection with VV(Cop)ΔF1L promoted the release of cytochrome c and caspase 3 activation (Figure 4-2B and C, panels c), indicating that F1L expression was essential during VV(Cop) infection to inhibit apoptosis induced during virus infection.

To determine if VV(Cop)ΔF1L infection initiated apoptosis we infected Jurkat cells with either VV(Cop) or VV(Cop)ΔF1L for 15 hours and assessed apoptosis by quantifying loss of the inner mitochondrial membrane potential by TMRE fluorescence (36). Mock infected and VV(Cop) infected Jurkat cells demonstrated 96% and 90% of the cells positive for TMRE fluorescence, respectively (Figure 4-3A panel a and b). In contrast, only 68% of the Jurkat cells infected with VV(Cop)ΔF1L demonstrated TMRE fluorescence with 32% of the cells showing a dramatic decrease in TMRE fluorescence indicative of cells undergoing apoptosis (Figure 4-3A panel c). Infection of Bcl-2 overexpressing Jurkat cells completely inhibited VV(Cop)ΔF1L induced apoptosis (Figure 4-3A panel f). To further assess the ability of VV(Cop)ΔF1L to initiate apoptosis in Jurkat cells we monitored both cytochrome c release and caspase 3 activation. Jurkat

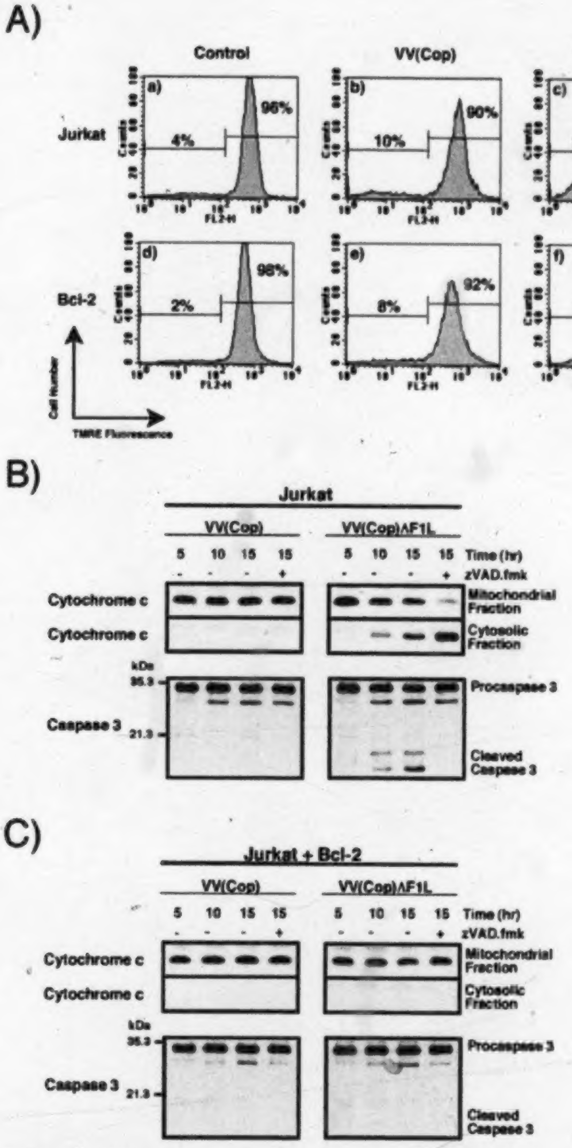


Figure 4-3 VV(Cop)ΔF1L induces apoptosis in Jurkat cells.
(A) Jurkat cells were infected with VV(Cop) or VV(Cop)ΔF1L at an MOI of 10 for 15 hours and apoptosis assessed by TMRE fluorescence. (B) Jurkat cells were infected with VV(Cop) or VV(Cop)ΔF1L at an MOI of 10 for 5, 10, and 15 hours. Cytochrome c release and caspase 3 activation were monitored by western blot analysis. (C) Bcl-2 overexpression is sufficient to inhibit VV(Cop)ΔF1L-induced apoptosis. Jurkat cells were infected with VV(Cop) or VV(Cop)ΔF1L at an MOI of 10 for 5, 10, and 15 hours in the presence and absence of zVAD.fmk. Cytochrome c release and caspase 3 activation were monitored by western blot analysis.

cells infected with VV(Cop) showed no cytochrome c release or caspase 3 activation (Figure 4-3B), whereas cells infected with VV(Cop)ΔF1L showed obvious release of mitochondria cytochrome c and caspase 3 cleavage at 10 and 15 hours post infection (Figure 4-3B). VV(Cop)ΔF1L-induced cytochrome c release occurred in the presence of the broad spectrum caspase inhibitor zVAD.fmk indicating that prior caspase activation was not a necessary requirement for VV(Cop)ΔF1L-induced cytochrome c release (Figure 4-3B). Additionally, the overexpression of Bcl-2 completely inhibited VV(Cop)ΔF1L-induced cytochrome c release and caspase 3 activation indicating that Bcl-2 compensated for the lack of F1L to inhibit VV(Cop)ΔF1L-induced apoptosis (Figure 4-3C).

Vaccinia Virus F1L Protein Specifically Interacts with Bak.

Our studies indicated that VV(Cop) inhibited apoptosis by regulating the mitochondrial checkpoint through the activity of the F1L open reading frame (47, 56, 57). F1L expression inhibits apoptosis by interfering with the release of cytochrome c and the loss of the inner mitochondrial membrane potential by an unknown mechanism (47, 57). Therefore, to determine the mechanism of action of F1L we asked if F1L functioned by interacting with members of the Bcl-2 family which tightly regulate the mitochondrial checkpoint in apoptotic cells (25, 44). Cell lysates from HeLa cells either mock infected or infected with a recombinant vaccinia virus expressing a Flag-tagged version of F1L, VV(WR)Flag-F1L, were applied to the anti-F1L immunoaffinity column. Flag-tagged F1L interacting proteins were subsequently eluted and potential F1L binding partners were monitored by western blotting. Using this approach, F1L successfully eluted as indicated by immunoblotting with an anti-Flag antibody (Figure 4-4). Both mock infected and vaccinia virus infected lysates displayed similar expression levels of Bax, Bak, Bcl-2, Bcl-XL and Mcl-1 whereas only the virus infected cell lysate expressed the Flag-tagged version of F1L (Figure 4-4). To determine if F1L interacted with members of the Bcl-2 family, the eluted fractions were probed with antibodies directed against various Bcl-2 family members. Under these conditions, the pro-apoptotic protein Bak consistently co-eluted with the Flag-tagged version of F1L while no interactions with Bax, Bcl-2, Bcl-XL or Mcl-1 were detected (Figure 4-4).

To further confirm the interaction between F1L and Bak, we performed a series of co-immunoprecipitation assays. HEK 293T cells were co-transfected with plasmids expressing EGFP-tagged F1L and HA-tagged Bak, lysed in 2% CHAPS and immunocomplexes were precipitated with either anti-EGFP or anti-HA antibodies. Immunoprecipitation with anti-EGFP resulted in co-immunoprecipitation of EGFP-F1L and HA-Bak (Figure 4-5A). Reciprocal immunoprecipitations performed with an anti-HA antibody also indicated that HA-Bak co-precipitated EGFP-F1L (Figure 4-5A). To determine the ability of F1L to interact with endogenous Bak in the absence of infection, HeLa cells were transfected with plasmids expressing EGFP or EGFP-F1L and immunoprecipitated with an anti-EGFP antibody. Both EGFP and EGFP-F1L immunoprecipitated with the anti-EGFP antibody (Figure 4-5B) and immunoblotting with an anti-Bak antibody indicated that endogenous Bak co-precipitated with F1L (Figure 4-5B). To confirm that F1L was able to interact with endogenous levels of Bak during infection, HeLa cells were infected with VV(Cop) and simultaneously transfected with either pSC66 or pSC66-Flag-F1L which places Flag-tagged F1L under the control

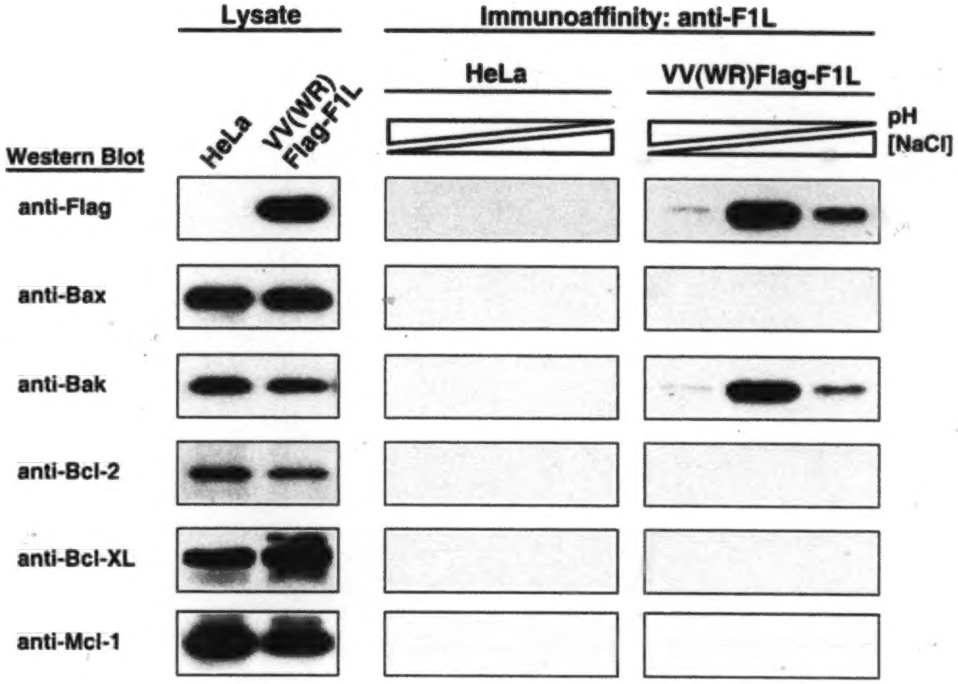

Lysate
Immunoaffinity: anti-F1L
HeLa
VV(WR)Flag-F1L
HeLa
VV(WR)Flag-F1L
pH
[NaCl]
Western Blot
anti-Flag
anti-Bax
anti-Bak
anti-Bcl-2
anti-Bcl-XL
anti-Mcl-1

Figure 4-4 F1L interacts with endogenous Bak but not other Bcl-2 family proteins. HeLa cell lysates from mock infected or infected with VV(WR)Flag-F1L at an MOI of 10 were applied on to an anti-F1L immunoaffinity column. Bound proteins were eluted by linear addition of elution buffer containing 100mM glycine and 0.5M NaCl pH 2.7. The eluted fractions were monitored by western blot for Flag-F1L, Bax, Bak, Bcl-2, Bcl-XL, and Mcl-1.

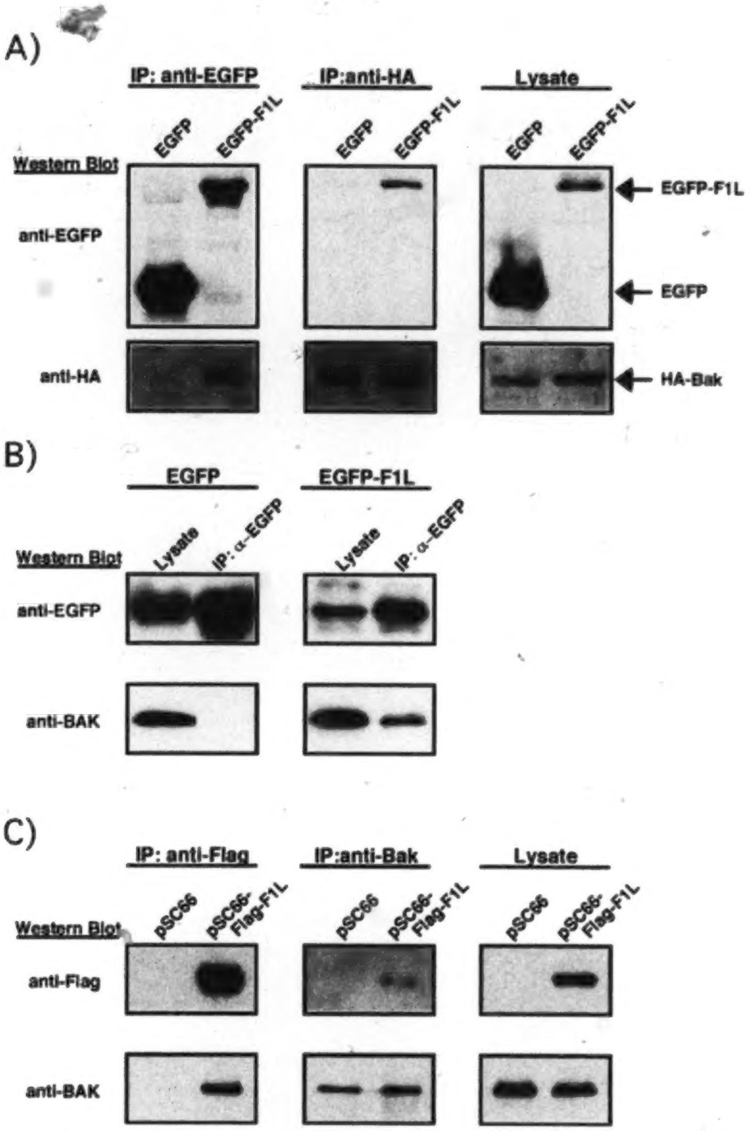

A)
IP: anti-EGFP
IP:anti-HA
Lysate
EGFP
EGFP-F1L
Western Blot
anti-EGFP
anti-HA
EGFP-F1L
EGFP
HA-Bak
B)
EGFP
EGFP-F1L
Lysate
IP: α-EGFP
Western Blot
anti-EGFP
anti-BAK
C)
IP: anti-Flag
IP:anti-Bak
Lysate
pSC66
pSC66-Flag-F1L
Western Blot
anti-Flag
anti-BAK

Figure 4-5 F1L interacts with Bak in the presence and absence of VV(Cop) infection.

(A) Ectopic expression of F1L and Bak demonstrates interaction between F1L and Bak. HEK 293T cells co-transfected with either pEGFP or pEGFP-F1L in the presence of pcDNA-HA-Bak. EGFP-F1L interacts with HA-Bak. (B) F1L interacts with endogenous Bak. HeLa cells were transfected with pEGFP or pEGFP-F1L. EGFP-F1L, but not EGFP, interacts with endogenous Bak. (C) F1L associates with endogenous Bak during virus infection. HeLa cells were infected with VV(Cop) at an MOI of 10 and transfected with pSC66 or pSC66-Flag-F1L to express Flag-F1L during infection. F1L associates with endogenous Bak.Experiment in Figure 4-5A performed by Logan Banadyga.

of a poxvirus promoter. Western blot analysis indicated that Flag-F1L was expressed only in cells transfected with pSC66-Flag-F1L and infected (Figure 4-5C) and antibodies directed against the Flag epitope effectively immunoprecipitated Flag-F1L and co-immunoprecipitated endogenous Bak (Figure 4-5C). The interaction between Flag-F1L and Bak during virus infection was confirmed by performing reciprocal co-immunoprecipitations (Figure 4-5C). To verify that F1L and Bak localized to the mitochondria during virus infection, HeLa cells were infected with VV(WR)Flag-F1L and the localization of Flag-F1L and endogenous Bak were monitored by confocal analysis. Using an antibody directed against the Flag epitope, cells infected with VV(WR)Flag-F1L demonstrated a punctate staining pattern (Figure 4-6, panel a) and a similar staining profile was detected for endogenous Bak (Figure 4-6, panel b). When the fluorescent signals generated from Flag-F1L and Bak were superimposed, co-localization was evident demonstrating that F1L and Bak localized to the mitochondria during virus infection (Figure 4-6 panel c).

F1L Inhibits Staurosporine-induced Bak Activation.

The interaction of F1L with Bak suggested that F1L may function by interfering with the pro-apoptotic activity of Bak. Following an apoptotic stimulus, Bak undergoes a multi-step activation process in which the N-terminus becomes exposed priming Bak for subsequent homo-oligomerization that results in the release of cytochrome c (24, 58). Therefore, we asked if infection with VV(Cop) affected Bak homo-oligomerization. Jurkat cells were either mock infected, infected with VV(Cop)-EGFP or VV(Cop)ΔF1L. Following treatment with staurosporine, whole cell lysates were solubilized in

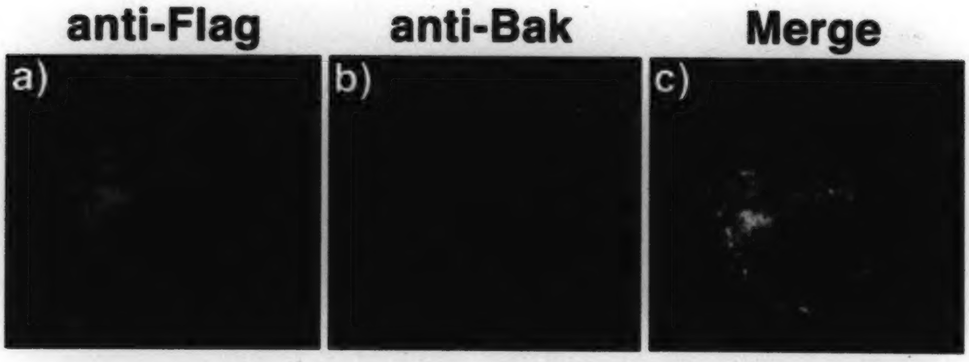


Figure 4-6 F1L and endogenous Bak localize at the mitochondria during vaccinia virus infection.
HeLa cells were infected with VV(WR)Flag-F1L at an MOI of 5 for 8 hours. The localization of Flag-F1L was visualized with a FITC-conjugated mouse anti-Flag antibody (panels a and c). Endogenous Bak was detected using an anti-Bak (G23) antibody followed by detection with the Alexa-Fluor 546-conjugated goat anti-rabbit antibody (panels b and c).

2%CHAPS buffer and Bak oligomerization was analyzed by gel filtration chromatography followed by immunoblotting with anti-Bak antibody. In the absence of staurosporine, Bak was detected in mock infected cells as an inactive form ranging in size from approximately 35kDa to 130kDa (Figure 4-7A). Treatment with staurosporine however resulted in loss of the lower molecular weight inactive form of Bak and the appearance of a higher molecular weight oligomeric form ranging from 200kDa to 360kDa (Figure 4-7A). When cells were infected with VV(Cop)-EGFP and treated with staurosporine, Bak oligomerization was clearly inhibited indicating that vaccinia virus infection interfered with Bak oligomerization (Figure 4-7A). In contrast to VV(Cop)-EGFP infected cells, the formation of higher molecular weight Bak oligomers was not inhibited when cells were infected with VV(Cop)ΔF1L and treated with staurosporine, indicating a role for F1L in the inhibition of Bak oligomerization (Figure 4-7A).

Bak oligomerization requires an initial conformational change which is characterized by exposure of the N-terminus (24, 43). To determine if vaccinia virus interfered with the initial conformational change in Bak, Jurkat cells were infected with VV(Cop)-EGFP or VV(Cop)ΔF1L and the activation of Bak was induced by the addition of staurosporine. The conformation of Bak was monitored using a conformation-specific anti-Bak antibody that recognizes the exposed N-terminus and activated Bak was detected by flow cytometry (24). As shown in Figure 4-7B, all untreated cells displayed low levels of background fluorescence associated with non-specific antibody staining. Jurkat cells treated with staurosporine and stained with anti-Bak resulted in a clear increase in fluorescence intensity indicating an increase in epitope availability for the conformation specific antibody (Figure 4-7B, panel a). No increase in fluorescence

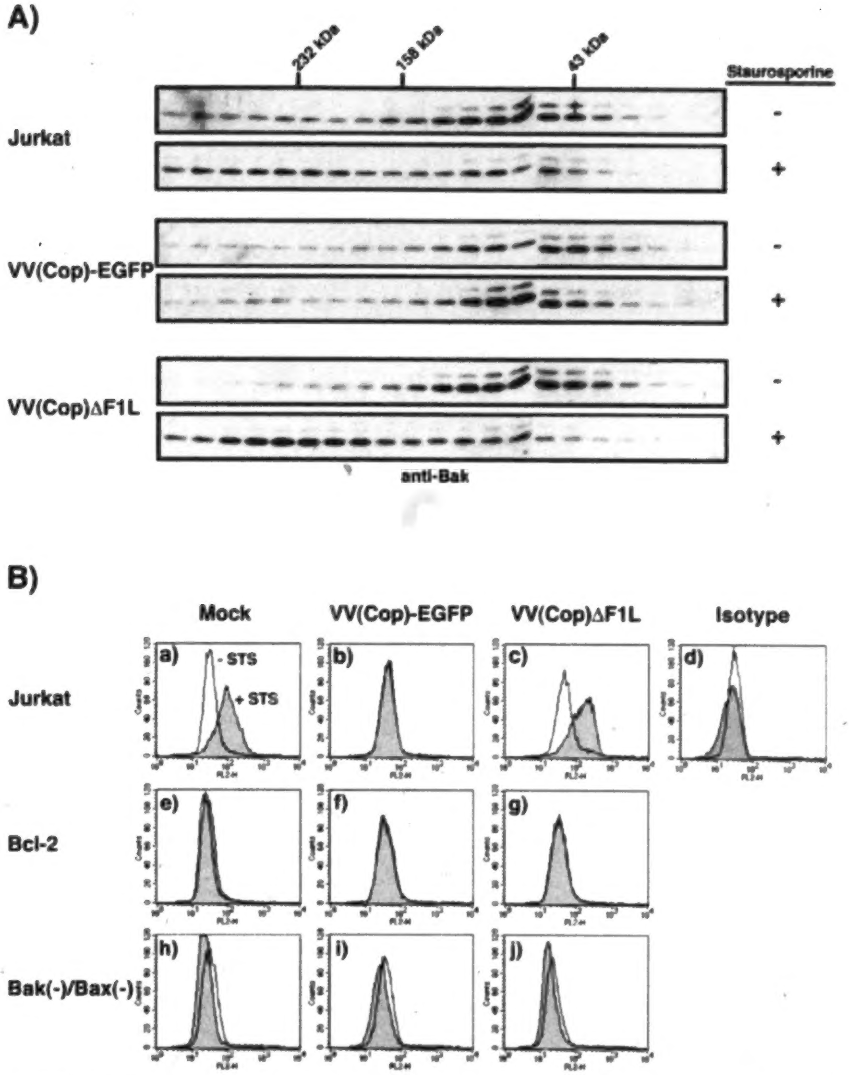


Figure 4-7 F1L expression inhibits staurosporine-induced Bak activation.
(A) Bak oligomerization is inhibited by VV(Cop)-EGFP infection but not infection with VV(Cop)ΔF1L. Jurkat cells were infected with VV(Cop)-EGFP or VV(Cop)ΔF1L at an MOI of 10 and 5 hours post infection treated with 1μM staurosporine for 2 hours to induce apoptosis. Bak oligomerization was assessed by gel filtration analysis and detected by western blot. (B) F1L expression is necessary for VV(Cop)-EGFP to inhibit the N-terminal exposure of Bak. Jurkat cells or Jurkat cells overexpressing Bcl-2 or Jurkat cells devoid of Bak and Bax were infected with VV(Cop)-EGFP or VV(Cop)ΔF1L at an MOI of 10 and treated with 250nM staurosporine for 2 hours to induce apoptosis. Exposure of the N-terminus of Bak was monitored by flow cytometry using a conformational specific N-terminal anti-Bak antibody or an isotype control antibody. Untreated cells (open histogram); staurosporine treated cells (shaded histogram).

intensity was detected in cells treated with staurosporine and stained with an isotype control antibody (Figure 4-7B panel d). As previously documented, Bcl-2 expression clearly inhibited the N-terminal exposure of Bak induced by staurosporine treatment (Figure 4-7B panel e)(24). To ensure we were measuring the N-terminal exposure of Bak, we performed similar experiments in Jurkat cells deficient in Bak and Bax (53). Staurosporine treated Jurkat cells deficient in Bak and Bax also demonstrated a lack of antibody staining clearly indicating the assay was measuring Bak activation (Figure 4-7B panel h-j). In contrast to mock infected Jurkat cells, infection with VV(Cop)-EGFP exhibited clear protection of the N-terminal exposure of Bak following staurosporine treatment (Figure 4-7B, panel b) while infection with VV(Cop)ΔF1L and the subsequent addition of staurosporine resulted in the appearance of N-terminal Bak epitope indicating that the presence of F1L was essential for vaccinia virus to inhibit staurosporine induced N-terminal Bak (Figure 4-7B panel c). The overexpression of Bcl-2 inhibited activation of Bak in cells infected with VV(Cop)ΔF1L indicating that Bcl-2 could functionally replace F1L (Figure 4-7B, panel g).

F1L Regulates tBid-induced Bak Activation.

Multiple apoptotic signals originating upstream of the mitochondria require Bak and Bax activation (25, 44, 59). A subclass of Bcl-2 family members which only contain BH3 domains such as Bid, initiate the activation of Bak and Bax (2, 58). Bid activation occurs through caspase 8 cleavage resulting in the C-terminal portion of Bid (tBid) to translocate to the mitochondria initiating the homo-oligomerization of Bak and Bax resulting in the release of cytochrome c (15, 33, 58). To determine the ability of F1L to modulate Bak activation induced by the BH3-only protein Bid, we utilized wild type MEFs, MEFs deficient in Bax and MEFs doubly deficient in Bak and Bax (59). Mitochondria purified from mock infected wild type MEFs and treated with increasing amounts of tBid resulted in the release cytochrome c into the supernatant fraction (Figure 4-8A panel a) which was completely inhibited in mitochondria purified from MEFs deficient in both Bak and Bax as previously documented (Figure 4-8A panel d) (34, 59). Purified mitochondria from VV(Cop) infected wildtype MEFs and treated with tBid were protected from cytochrome c release (Figure 4-8A panel b) while mitochondria purified from wildtype MEFs infected with VV(Cop)ΔF1L were unable to inhibit cytochrome c release (Figure 4-8A panel c). In fact, when compared to mock infected conditions, infection with VV(Cop)ΔF1L resulted in mitochondria that were more sensitive to tBid as indicated by the requirement of lower amounts of tBid to achieve cytochrome c release (Figure 4-8A panel c). These results indicated that VV(Cop) infection inhibited tBid induced cytochrome c release mediated by Bak and Bax and that F1L was necessary for this inhibitory effect. Additionally, we infected Bax deficient MEFs which allowed us to exclude the pro-apoptotic function of Bax and focus specifically on Bak. VV(Cop) infection of MEFs deficient in Bax also provided protection from tBid-induced cytochrome c release (Figure 4-8A panel e) and the expression of F1L was necessary to inhibit Bak induced release of cytochrome c (Figure 4-8A panel f).

To determine if F1L inhibited tBid induced Bak homo-oligomerization, we monitored the appearance of higher order Bak complexes. Mitochondria were isolated from MEFs deficient in Bax, treated with tBid and Bak oligomerization was monitored by chemical crosslinking with BMH and assessed by western blot. In the absence of

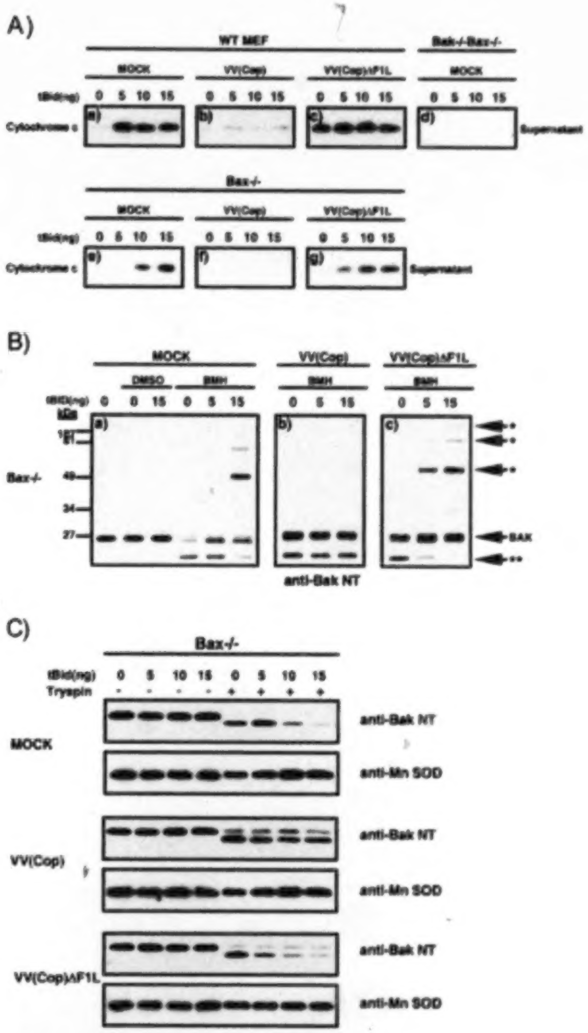

A)
WT MEF
Bak-/-Bax-/-
MOCK
VV(Cop)
VV(Cop)ΔF1L
MOCK
tBid(ng) 0 5 10 15 0 5 10 15 0 5 10 15 0 5 10 15
a)
b)
c)
d)
Cytochrome c
Supernatant
Bax-/-
MOCK
VV(Cop)
VV(Cop)ΔF1L
tBid(ng) 0 5 10 15 0 5 10 15 0 5 10 15
e)
f)
g)
Cytochrome c
Supernatant
B)
MOCK
DMSO
BMH
VV(Cop)
BMH
VV(Cop)ΔF1L
BMH
tBid(ng) 0 0 15 0 5 15 0 5 15 0 5 15
kDa
49
34
27
Bax-/-
a)
b)
c)
*
*
*
BAK
**
anti-Bak NT
C)
Bax-/-
tBid(ng) 0 5 10 15 0 5 10 15
Trypsin - - - - + + + +
MOCK
anti-Bak NT
anti-Mn SOD
VV(Cop)
anti-Bak NT
anti-Mn SOD
VV(Cop)ΔF1L
anti-Bak NT
anti-Mn SOD

Figure 4-8 F1L inhibits tBid-induced Bak activation.
(A) F1L expression inhibits release of cytochrome c initiated by tBid. Mitochondria were isolated from wildtype MEFs, Bax deficient MEFs, and Bax and Bak deficient MEFs that had previously been infected with VV(Cop) or VV(Cop)ΔF1L at an MOI of 10. Mitochondria were treated with increasing amounts of tBid and assessed for cytochrome c release by western blot. (B) F1L expression is necessary to inhibit Bak oligomerization initiated by tBid. Mitochondria from Bax deficient MEFs were treated with tBid and crosslinked with BMH. VV(Cop)EGFP infection but not VV(Cop)ΔF1L infection inhibits Bak oligomerization. "*"Bak homo-oligomers; "**"monomeric intramolecular-crosslinked Bak species. (C)F1L expression is necessary to inhibit the N-terminal exposure of Bak induced by increasing amounts of tBid. After exposure to tBid, isolated mitochondria were treated with trypsin and Bak conformation was monitored by western blot. As a control the presence of Mn SOD was also monitored by western blot.

BMH, Bak migrates at approximately 26kDa (Figure 4-8B panel a), however, upon the addition of BMH, Bak displays a faster mobility as a result of intramolecular-crosslinking (Figure 4-8B) (43, 58). The addition of tBid to mitochondria isolated from Bax deficient MEFs resulted in loss of the intramolecular crosslinked Bak species and the formation of Bak oligomers indicative of Bak activation (Figure 4-8B panel a) (43, 59). Mitochondria purified from Bax deficient MEFs infected with VV(Cop) and treated with tBid showed retention of the intramolecular-crosslinked species of Bak and the absence of Bak homo-oligomers (Figure 4-8B panel b). In contrast, Bak oligomerization and loss of the intramolecular-crosslinked Bak species were detected in mitochondria isolated from VV(Cop)ΔF1L infected Bax deficient MEFs and treated with tBid indicating that F1L was necessary to inhibit tBid induced Bak oligomerization (Figure 4-8B panel c). Moreover, infection with VV(Cop)ΔF1L appeared to augment the efficacy of tBid to promote homo-oligomerization of Bak (Figure 4-8B panel c).

To determine if F1L was capable of inhibiting the initial tBid-induced activation of Bak, Bak conformation was assessed by limited proteolysis. In an inactive state, the N-terminus of Bak is inaccessible to trypsin proteolysis, however, following an apoptotic trigger, the N-terminus of Bak becomes exposed and susceptible to trypsin-mediated proteolysis (43, 58). As such, Bak conformational changes can be monitored as a loss of antibody reactivity during western blot analysis using an antibody specific for the N-terminus of Bak (43, 58). In the absence of trypsin, all mitochondria displayed equal levels of Bak as measured by western blot analysis (Figure 4-8C). Following treatment with trypsin, mitochondria isolated from mock infected Bax deficient MEFs and incubated with tBid resulted in an increase in susceptibility of Bak to proteolysis as detected by the appearance of a lower molecular weight form of Bak and the eventual loss of antibody reactivity with increasing amounts of tBid (Figure 4-8C). Western blot analysis of the mitochondrial matrix protein manganese superoxide dismutase (Mn SOD) served as a loading control (Figure 4-8C). Mitochondria isolated from VV(Cop) infected MEFs deficient in Bax and treated with tBid showed clear retention of trypsin resistant Bak (Figure 4-8C). However, Bak sensitivity to proteolysis was observed in mitochondria isolated from Bax deficient MEFs infected with VV(Cop)ΔF1L (Figure 4-8C) indicating that VV(Cop) infection inhibited the conformational change of Bak induced by tBid and that F1L was essential for this inhibition.

4.4 Discussion

To ensure successful propagation, viruses must overcome the host innate and acquired immune responses (50). Members of the Poxviridae family, including vaccinia virus the prototypic member of the family, encode numerous immunomodulatory proteins to counteract host anti-viral strategies including apoptosis (45, 46). Apoptosis is a characteristic form of cellular suicide that can be initiated by a wide variety of stimuli resulting in the ultimate destruction of the cell (27). Members of the poxvirus family have evolved a wide range of strategies to interfere with apoptosis in order to ensure efficient virus propagation and dissemination (4, 18). We recently identified F1L as an additional anti-apoptotic protein encoded by vaccinia virus (47, 57). F1L is a tail-anchored protein that localizes to the outer mitochondria membrane and inhibits the release of cytochrome c and loss of the inner mitochondrial membrane potential by an unknown mechanism (47, 57). We now provide evidence that F1L expression inhibits the activation of Bak, a pro-apoptotic member of the Bcl-2 family.

To further understand the anti-apoptotic mechanism of F1L protein, we set out to identify potential cellular partners for F1L. We focused on the members of the Bcl-2 family which tightly regulate the apoptotic cascade at the mitochondria. Using an anti-F1L affinity column we found that F1L interacted with the pro-apoptotic Bcl-2 family member Bak, but not with the pro-apoptotic protein Bax, or with the anti-apoptotic proteins Bcl-2, Bcl-XL or Mcl-1. The interaction between F1L and Bak was confirmed by co-immunoprecipitation which clearly demonstrated that F1L interacted with endogenous Bak in the absence and presence of infection. Bak is a pro-apoptotic member of the Bcl-2 family that constitutively localizes to the outer mitochondrial membrane playing a pivotal role in cytochrome c release from mitochondria (34, 58, 59).

During virus infection F1L interacts constitutively with Bak and the expression of F1L is essential to inhibit the release of cytochrome c suggesting that F1L may function by interfering with the pro-apoptotic function of Bak. The activation of multidomain pro-apoptotic proteins Bak and Bax constitutes a critical step in the release of cytochrome c that is antagonized by anti-apoptotic Bcl-2 family members as well as virus encoded proteins (25, 44, 59). In response to an apoptotic stimulus, Bak undergoes a conformational change exposing a new epitope followed by Bak homo-oligomerization (24, 58). Using gel filtration analysis, we found that vaccinia virus infection potently prevented Bak homo-oligomerization induced by staurosporine and F1L was essential for this inhibition. Flow cytometric data using a conformational specific Bak antibody also indicated that F1L blocked staurosporine-induced N-terminal exposure of Bak.

Bak and Bax activation is regulated by a subset of Bcl-2 family members referred to as BH3-only proteins which are activated by postranslational modification, transcriptional upregualtion, or caspase activation (48). Caspase 8 cleavage of the BH3-only protein Bid generates a C-terminal fragment referred to as tBid that can directly activate Bak and Bax (31, 43, 58). We used recombinant tBid to test the ability of vaccinia virus and F1L to regulate the direct activation of Bak and Bax by tBid. Our data indicated that vaccinia virus infection inhibited tBid induced cytochrome c release from mitochondria purified from both wildtype MEFs and Bax deficient MEFs. The expression of F1L was absolutely essential to inhibit tBid induced cytochrome c release from both cell lines suggesting that F1L could inhibit direct activation of Bak and Bax by tBid. Using crosslinking and limited trypic digests, we found that F1L expression during infection prevented tBid induced Bak oligomerization and the conformational change in Bak.

Several potential mechanisms can be envisioned for F1L inhibition of Bak. The simplest explanation would be that F1L directly interacts with Bak preventing its activation. A similar role for VDAC2 and Mcl-1 has been described in which both cellular proteins sequester Bak in an inactive conformation (9, 12). Alternatively, F1L could augment the activity of VDAC2 and Mcl-1 to keep Bak in an inactive state (9, 12). Following an apoptosis stimulus Mcl-1 is released from Bak allowing for the activation of Bak and the subsequent destruction of Mcl-1 by the 26S proteasome (12, 32, 37). We were unable to detect an interaction between F1L and Mcl-1 (Figure 4-4) suggesting that in the presence of F1L, Mcl-1 no longer interacts with Bak resulting in the degradation of Mcl-1 following vaccinia virus infection, a possibility we are pursuing. Additionally, our data clearly demonstrated that F1L inhibits apoptosis in cells that express both Bak and Bax suggesting that F1L may inhibit Bax activation. Bak and Bax activation is initiated either directly or indirectly by BH3-only proteins such as Bid and it is well established that anti-apoptotic Bcl-2 proteins sequester BH3-only proteins to disrupt Bak and Bax activation (25, 44). Therefore, F1L may function by sequestering and inhibiting the activity of BH3-only proteins in a similar fashion proposed for Bcl-2 (8, 10, 20, 30, 48). Through this mechanism F1L expression would ultimately inhibit activation of both Bak and Bax.

The regulation of Bak and Bax is a common theme employed by viruses. Many viruses encode obvious Bcl-2 homologues that function by inhibiting Bak and Bax activation (11, 26, 40). E1B19K, encoded by adenovirus, interacts with both Bak and Bax following N-terminal exposure of both proteins (11, 49). VMIA, encoded by HCMV, paradoxically recruits Bax to mitochondria and freezes the homo-oligomeric configuration of Bax (3, 41). Recently, M11L, encoded by myxoma virus, another member of the poxvirus family, was found to constitutively interact with Bak and prevent apoptosis by an undefined mechanism (52). Notably, F1L open reading frames are only present in members of the Orthopoxvirus genera while members of the Leporipoxviruses, Capripoxvirus, Suipoxvirus and Yatapoxvirus genera encode M11L which function to inhibit apoptosis at the mitochondria. In contrast, the Avipoxviruses are to date the only members of the poxvirus family to encode obvious Bcl-2 family members (1, 51). Although M11L and F1L display no obvious sequence homology, both localize to the mitochondria and inhibit the release of cytochrome c. Data generated in our laboratory clearly shows that the Bcl-2 homologue in fowlpox virus also functions at the mitochondria to inhibit apoptosis (L. Banadyga and M. Barry unpublished data). As such, members of the poxvirus family have evolved distinct proteins that are functionally conserved to inhibit apoptosis. The modulation of Bak and Bax during poxvirus infection reflects the important role of the mitochondria in the elimination of poxvirus-infected cells. In support of this, we found that vaccinia virus lacking F1L induced an intrinsic apoptotic cascade that could be inhibited by overexpression of Bcl-2. A similar observation was recently made using modified vaccinia virus Ankara missing F1L (20). Vaccinia virus-induced apoptosis correlated with Bak activation and oligomerization and the release of cytochrome c. The release of cytochrome c induced by virus infection was caspase independent suggesting the involvement of caspase-independent BH3-only proteins as initiators of the apoptotic cascade. Although we did not observe a growth defect upon infection of CV-1 cells infected with vaccinia virus missing F1L, regulation of the mitochondrial checkpoint to ensure successful viral replication has been observed with other viruses. For example, adenovirus relies on the expression of E1B 19K to inhibit apoptosis by regulating Bak and Bax (11). Similar results have been reported for HCMV. In the absence of vMIA expression, HCMV induces apoptosis that results in inefficient viral replication (42). Additionally, myxoma virus deleted in M11L induces apoptosis in primary monocytes and rabbit lymphocytes (RL-5 cells)(16, 35). A growth defect for the M11L deficient virus was noted in RL-5 cells and spleen cells but not in rabbit SIRC cells (35, 39). The lack of a restricted growth phenotype in CV-1 cells following infection with VV(Cop)ΔF1L suggested that the F1L deficient virus was unable to induce apoptosis in these cells. In support of this, infection of CV-1 cells and BGMK cells with VV(Cop)ΔF1L resulted in limited amounts of apoptosis at 24 hours post infection as measured by TUNEL assay (data not shown). We speculate that apoptosis induced by the F1L deletion virus may evoke a growth defect in other cell lines other than CV-1 and BGMK cells or in primary cells, as documented for the anti-apoptotic protein M11L (35, 39).

The importance of modulating the mitochondria to control apoptosis is a general strategy employed by viruses. The observation that F1L interacts with Bak and regulates Bak function highlights the importance of Bak in regulating apoptosis and the necessity to maintain a suitable cellular environment during virus infection. Understanding the complexities of the interaction between Bak and F1L will provide important clues regarding the mechanism of cytochrome c release and mitochondrial permeabilization.

4.5 References

1. **Afonso, C. L., E. R. Tulman, Z. Lu, L. Zsak, G. F. Kutish, and D. L. Rock.** 2000. The genome of fowlpox virus. J Virol **74:**3815-31.
2. **Antonsson, B., S. Montessuit, B. Sanchez, and J. C. Martinou.** 2001. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem **276:**11615-23.
3. **Arnoult, D., L. M. Bartle, A. Skaletskaya, D. Poncet, N. Zamzami, P. U. Park, J. Sharpe, R. J. Youle, and V. S. Goldmacher.** 2004. Cytomegalovirus cell death suppressor vMIA blocks Bax- but not Bak-mediated apoptosis by binding and sequestering Bax at mitochondria. Proc Natl Acad Sci U S A **101:**7988-93.
4. **Barry, M., S. T. Wasilenko, T. L. Stewart, and J. M. Taylor.** 2004. Apoptosis regulator genes encoded by poxviruses. Prog Mol Subcell Biol **36:**19-37.
5. **Boya, P., A. L. Pauleau, D. Poncet, R. A. Gonzalez-Polo, N. Zamzami, and G. Kroemer.** 2004. Viral proteins targeting mitochondria: controlling cell death. Biochim Biophys Acta **1659:**178-89.
6. **Boya, P., B. Roques, and G. Kroemer.** 2001. New EMBO members' review: viral and bacterial proteins regulating apoptosis at the mitochondrial level. Embo J **20:**4325-31.
7. **Cartron, P. F., H. Arokium, L. Oliver, K. Meflah, S. Manon, and F. M. Vallette.** 2005. Distinct domains control the addressing and the insertion of Bax into mitochondria. J Biol Chem **280:**10587-98.
8. **Chen, L., S. N. Willis, A. Wei, B. J. Smith, J. I. Fletcher, M. G. Hinds, P. M. Colman, C. L. Day, J. M. Adams, and D. C. Huang.** 2005. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell **17:**393-403.
9. **Cheng, E. H., T. V. Sheiko, J. K. Fisher, W. J. Craigen, and S. J. Korsmeyer.** 2003. VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science **301:**513-7.
10. **Cory, S., D. C. Huang, and J. M. Adams.** 2003. The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene **22:**8590-607.
11. **Cuconati, A., K. Degenhardt, R. Sundararajan, A. Anschel, and E. White.** 2002. Bak and Bax function to limit adenovirus replication through apoptosis induction. J Virol **76:**4547-58.
12. **Cuconati, A., C. Mukherjee, D. Perez, and E. White.** 2003. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. Genes Dev **17:**2922-32.
13. **Cuconati, A., and E. White.** 2002. Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. Genes Dev **16:**2465-78.
14. **Earl, P. L., Moss, B., Wyatt, L.S., Carroll, M.W.** 1998. Generation of recombinant vaccinia viruses., p. 16.17.1– 16.17.19. *In* F. M. Ausubel, Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (ed.), Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley Interscience, New York.
15. **Eskes, R., S. Desagher, B. Antonsson, and J. C. Martinou.** 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol **20:**929-35.
16. **Everett, H., M. Barry, S. F. Lee, X. Sun, K. Graham, J. Stone, R. C. Bleackley, and G. McFadden.** 2000. M11L: a novel mitochondria-localized protein of myxoma virus that blocks apoptosis of infected leukocytes. J Exp Med **191:**1487-98.
17. **Everett, H., M. Barry, X. Sun, S. F. Lee, C. Frantz, L. G. Berthiaume, G. McFadden, and R. C. Bleackley.** 2002. The Myxoma Poxvirus Protein, M11L, Prevents Apoptosis by Direct Interaction with the Mitochondrial Permeability Transition Pore. J Exp Med **196:**1127-1140.
18. **Everett, H., and G. McFadden.** 2002. Poxviruses and apoptosis: a time to die. Curr Opin Microbiol **5:**395.
19. **Feng, P., J. Park, B. S. Lee, S. H. Lee, R. J. Bram, and J. U. Jung.** 2002. Kaposi's sarcoma-associated herpesvirus mitochondrial K7 protein targets a cellular calcium-modulating cyclophilin ligand to modulate intracellular calcium concentration and inhibit apoptosis. J Virol **76:**11491-504.
20. **Fischer, S. F., H. Ludwig, J. Holzapfel, M. Kvansakul, L. Chen, D. C. Huang, G. Sutter, M. Knese, and G. Hacker.** 2005. Modified vaccinia virus Ankara protein F1L is a novel BH3-

domain-binding protein and acts together with the early viral protein E3L to block virus-associated apoptosis. Cell Death Differ.

21. **Goldmacher, V. S., L. M. Bartle, A. Skaletskaya, C. A. Dionne, N. L. Kedersha, C. A. Vater, J. W. Han, R. J. Lutz, S. Watanabe, E. D. Cahir McFarland, E. D. Kieff, E. S. Mocarski, and T. Chittenden.** 1999. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc Natl Acad Sci U S A **96:**12536-41.

22. **Green, D. R., and G. Kroemer.** 2004. The pathophysiology of mitochondrial cell death. Science **305:**626-9.

23. **Griffiths, G. J., B. M. Corfe, P. Savory, S. Leech, M. D. Esposti, J. A. Hickman, and C. Dive.** 2001. Cellular damage signals promote sequential changes at the N-terminus and BH-1 domain of the pro-apoptotic protein Bak. Oncogene **20:**7668-76.

24. **Griffiths, G. J., L. Dubrez, C. P. Morgan, N. A. Jones, J. Whitehouse, B. M. Corfe, C. Dive, and J. A. Hickman.** 1999. Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. J Cell Biol **144:**903-14.

25. **Gross, A., J. M. McDonnell, and S. J. Korsmeyer.** 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev **13:**1899-911.

26. **Hardwick, J. M., and D. S. Bellows.** 2003. Viral versus cellular BCL-2 proteins. Cell Death Differ **10 Suppl 1:**S68-76.

27. **Hengartner, M. O.** 2000. The biochemistry of apoptosis. Nature **407:**770-6.

28. **Koo, G. C., and J. R. Peppard.** 1984. Establishment of monoclonal anti-Nk-1.1 antibody. Hybridoma **3:**301-3.

29. **Kroemer, G., and J. C. Reed.** 2000. Mitochondrial control of cell death. Nat Med **6:**513-9.

30. **Kuwana, T., L. Bouchier-Hayes, J. E. Chipuk, C. Bonzon, B. A. Sullivan, D. R. Green, and D. D. Newmeyer.** 2005. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol Cell **17:**525-35.

31. **Kuwana, T., M. R. Mackey, G. Perkins, M. H. Ellisman, M. Latterich, R. Schneiter, D. R. Green, and D. D. Newmeyer.** 2002. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell **111:**331-42.

32. **Leu, J. I., P. Dumont, M. Hafey, M. E. Murphy, and D. L. George.** 2004. Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol **6:**443-50.

33. **Li, H., H. Zhu, C. J. Xu, and J. Yuan.** 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell **94:**491-501.

34. **Lindsten, T., A. J. Ross, A. King, W. X. Zong, J. C. Rathmell, H. A. Shiels, E. Ulrich, K. G. Waymire, P. Mahar, K. Frauwirth, Y. Chen, M. Wei, V. M. Eng, D. M. Adelman, M. C. Simon, A. Ma, J. A. Golden, G. Evan, S. J. Korsmeyer, G. R. MacGregor, and C. B. Thompson.** 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell **6:**1389-99.

35. **Macen, J. L., K. A. Graham, S. F. Lee, M. Schreiber, L. K. Boshkov, and G. McFadden.** 1996. Expression of the myxoma virus tumor necrosis factor receptor homologue and M11L genes is required to prevent virus-induced apoptosis in infected rabbit T lymphocytes. Virology **218:**232-7.

36. **Metivier, D., B. Dallaporta, N. Zamzami, N. Larochette, S. A. Susin, I. Marzo, and G. Kroemer.** 1998. Cytofluorometric detection of mitochondrial alterations in early CD95/Fas/APO-1-triggered apoptosis of Jurkat T lymphoma cells. Comparison of seven mitochondrion-specific fluorochromes. Immunol Lett **61:**157-63.

37. **Nijhawan, D., M. Fang, E. Traer, Q. Zhong, W. Gao, F. Du, and X. Wang.** 2003. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev **17:**1475-86.

38. **Opferman, J. T., and S. J. Korsmeyer.** 2003. Apoptosis in the development and maintenance of the immune system. Nat Immunol **4:**410-5.

39. **Opgenorth, A., K. Graham, N. Nation, D. Strayer, and G. McFadden.** 1992. Deletion analysis of two tandemly arranged virulence genes in myxoma virus, M11L and myxoma growth factor. J Virol **66:**4720-31.

40. **Polster, B. M., J. Pevsner, and J. M. Hardwick.** 2004. Viral Bcl-2 homologs and their role in virus replication and associated diseases. Biochim Biophys Acta **1644:**211-27.

41. **Poncet, D., N. Larochette, A. L. Pauleau, P. Boya, A. A. Jalil, P. F. Cartron, F. Vallette, C. Schnebelen, L. M. Bartle, A. Skaletskaya, D. Boutolleau, J. C. Martinou, V. S. Goldmacher, G. Kroemer, and N. Zamzami.** 2004. An anti-apoptotic viral protein that recruits Bax to mitochondria. J Biol Chem **279:**22605-14.
42. **Reboredo, M., R. F. Greaves, and G. Hahn.** 2004. Human cytomegalovirus proteins encoded by UL37 exon 1 protect infected fibroblasts against virus-induced apoptosis and are required for efficient virus replication. J Gen Virol **85:**3555-67.
43. **Ruffolo, S. C., and G. C. Shore.** 2003. BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization. J Biol Chem **278:**25039-45.
44. **Scorrano, L., and S. J. Korsmeyer.** 2003. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun **304:**437-44.
45. **Seet, B. T., J. B. Johnston, C. R. Brunetti, J. W. Barrett, H. Everett, C. Cameron, J. Sypula, S. H. Nazarian, A. Lucas, and G. McFadden.** 2003. Poxviruses and immune evasion. Annu Rev Immunol **21:**377-423.
46. **Smith, G. L., J. A. Symons, A. Khanna, A. Vanderplasschen, and A. Alcami.** 1997. Vaccinia virus immune evasion. Immunol Rev **159:**137-54.
47. **Stewart, T. L., S. T. Wasilenko, and M. Barry.** 2005. Vaccinia virus F1L protein is a tail-anchored protein that functions at the mitochondria to inhibit apoptosis. J Virol **79:**1084-98.
48. **Strasser, A.** 2005. The role of BH3-only proteins in the immune system. Nat Rev Immunol **5:**189-200.
49. **Sundararajan, R., A. Cuconati, D. Nelson, and E. White.** 2001. Tumor necrosis factor-alpha induces Bax-Bak interaction and apoptosis, which is inhibited by adenovirus E1B 19K. J Biol Chem **276:**45120-7.
50. **Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh.** 2000. Viral subversion of the immune system. Annu Rev Immunol **18:**861-926.
51. **Tulman, E. R., C. L. Afonso, Z. Lu, L. Zsak, G. F. Kutish, and D. L. Rock.** 2004. The genome of canarypox virus. J Virol **78:**353-66.
52. **Wang, G., J. W. Barrett, S. H. Nazarian, H. Everett, X. Gao, C. Bleackley, K. Colwill, M. F. Moran, and G. McFadden.** 2004. Myxoma virus M11L prevents apoptosis through constitutive interaction with Bak. J Virol **78:**7097-111.
53. **Wang, G. Q., E. Wieckowski, L. A. Goldstein, B. R. Gastman, A. Rabinovitz, A. Gambotto, S. Li, B. Fang, X. M. Yin, and H. Rabinowich.** 2001. Resistance to granzyme B-mediated cytochrome c release in Bak-deficient cells. J Exp Med **194:**1325-37.
54. **Wang, H. W., T. V. Sharp, A. Koumi, G. Koentges, and C. Boshoff.** 2002. Characterization of an anti-apoptotic glycoprotein encoded by Kaposi's sarcoma-associated herpesvirus which resembles a spliced variant of human survivin. Embo J **21:**2602-15.
55. **Wang, X.** 2001. The expanding role of mitochondria in apoptosis. Genes Dev **15:**2922-33.
56. **Wasilenko, S. T., A. F. Meyers, K. Vander Helm, and M. Barry.** 2001. Vaccinia virus infection disarms the mitochondrion-mediated pathway of the apoptotic cascade by modulating the permeability transition pore. J Virol **75:**11437-48.
57. **Wasilenko, S. T., T. L. Stewart, A. F. Meyers, and M. Barry.** 2003. Vaccinia virus encodes a previously uncharacterized mitochondrial-associated inhibitor of apoptosis. Proc Natl Acad Sci U S A **100:**14345-50.
58. **Wei, M. C., T. Lindsten, V. K. Mootha, S. Weiler, A. Gross, M. Ashiya, C. B. Thompson, and S. J. Korsmeyer.** 2000. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev **14:**2060-71.
59. **Wei, M. C., W. X. Zong, E. H. Cheng, T. Lindsten, V. Panoutsakopoulou, A. J. Ross, K. A. Roth, G. R. MacGregor, C. B. Thompson, and S. J. Korsmeyer.** 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science **292:**727-30.
60. **Wolter, K. G., Y. T. Hsu, C. L. Smith, A. Nechushtan, X. G. Xi, and R. J. Youle.** 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol **139:**1281-92.
61. **Zong, W. X., C. Li, G. Hatzivassiliou, T. Lindsten, Q. C. Yu, J. Yuan, and C. B. Thompson.** 2003. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol **162:**59-69.

Chapter 5: Discussion

5.1 Summary of Thesis

At the time I began my graduate degree, apoptosis was an area of intense research (41). As of September 1999, twenty-nine thousand apoptosis-related research articles were published, with nearly 90% of these publications occurring between 1995 and 1999. This robust explosion of research rapidly advanced our understanding of apoptosis and led to many exciting discoveries, one of which was the importance of the mitochondria during cell death (20, 29, 44). From a virology point of view, the importance of the mitochondria during apoptosis suggested that this organelle would be an important site for viruses to regulate apoptosis. True to form, many viruses encode Bcl-2 like proteins while other viruses encode novel anti-apoptotic proteins that function at the mitochondria (19, 23, 27, 32). The importance of mitochondria during apoptosis and the identification of anti-apoptotic virus-encoded proteins that function at mitochondria led us to hypothesize that vaccinia virus would inhibit apoptosis at the mitochondria. Prior to our studies, the majority of the anti-apoptotic activity provided by vaccinia virus was attributed to the function of CrmA/Spi-2 (21, 42). The literature however, contained subtle hints that vaccinia virus encoded other mechanisms to inhibit apoptosis (21, 42). To test our hypothesis, we took advantage of Jurkat cells, which undergo Fas-mediated apoptosis exclusively through the mitochondria, and infected these cells with vaccinia virus strain Copenhagen which is naturally devoid of a functional CrmA/Spi-2 protein (26, 65). Using this approach, we found that vaccinia virus infection prevented apoptosis by regulating the mitochondrial checkpoint (83, 84). These findings served as a platform for future studies.

The complete genome sequence of vaccinia virus strain Copenhagen was published in 1990 (26). Analysis of the genome failed to identify any open reading frames with homology to known mitochondrial anti-apoptotic proteins suggesting that vaccinia virus encoded a novel inhibitor of apoptosis. To identify the virus gene(s) responsible for the inhibition of apoptosis at the mitochondria, we utilized two vaccinia virus deletion viruses, VV759, which is missing 18 open reading frames, and VV811, which is missing 55 open reading frames, and asked if infection of Jurkat cells with these viruses inhibited apoptosis induced by external stimuli (57). We found that VV759 infection, but not VV811 infection, protected Jurkat cells from the hallmark features of apoptosis (Figure 3-2 and 3-3). Importantly, this data indicated that VV811 was missing an open reading frame(s) necessary to inhibit apoptosis and therefore, permitted the exclusion of a number of proteins in our search for a novel anti-apoptotic protein encoded by the virus. To further focus our search, we performed a peptide based blast search screening vaccinia virus open reading frames for a hydrophobic C-terminal mitochondrial targeting motif present in anti-apoptotic proteins such as Bcl-2, Bcl-XL and M11L (1, 23). This approach identified five candidate proteins that each contained a putative C-terminal mitochondrial targeting motif. The F1L open reading frame was particularly interesting since it was the only open reading frame containing a putative mitochondrial targeting motif that was noticeably absent in VV811. Taking advantage of the inability of VV811 to inhibit apoptosis, we expressed F1L during VV811 infection and found that F1L expression inhibited the loss of the inner mitochondrial membrane potential induced by staurosporine and anti-Fas (Figure 3-4). Follow-up studies demonstrated that F1L localized to mitochondria when expressed ectopically as well as during virus infection

(Figure 3-5). Additionally, mitochondrial localization of F1L was found to be dependent on the putative C-terminal targeting motif (Figure 3-7) (76). Further experiments indicated that expression of F1L, in the absence of vaccinia virus infection, inhibited mitochondrial-associated changes induced during apoptosis including the loss of the inner mitochondrial membrane potential and the release of cytochrome c and SMAC/Diablo thereby establishing F1L as a bona fide mitochondrial-associated inhibitor of apoptosis (Figure 3-9).

With the exception of Molluscipoxviruses, all *Chordopoxvirinae* genomes sequenced thus far contain a putative mitochondrial inhibitor of apoptosis. Members of the Avipoxvirus genus encode an obvious Bcl-2 family member (2, 80). The M11L open reading frame which confers resistance to apoptosis is present in Leporipoxvirus, Capripoxvirus, Suipoxvirus, and Yatapoxvirus genera while F1L open reading frames are exclusively found in members of the Orthopoxvirus genera. Sequence alignment of the F1L orthologs indicates that all orthologs are highly conserved (greater than 90%) within the last 226 amino acids of the protein (Figure 3-7). Interestingly, F1L orthologs contain a unique N-terminal region that is characterized by a varying number of amino acid repeats. With the exception of ectromelia virus, the N-terminal repeat regions in other members of the orthopoxvirus genus are comprised of a series of DDI amino acid repeats. In contrast, the N-terminal region of the ectromelia virus F1L ortholog contains an eight amino acid repeat (NGIVQDID) that extends for nearly 220 amino acids. The function of the N-terminal repeat region is currently unknown however, evidence indicates that it is not necessary for the inhibition of apoptosis, since vaccinia virus strain Copenhagen encoded F1L displays anti-apoptotic function and contains only a single DDI repeat motif and ectromelia virus F1L devoid of its N-terminal extension in the absence of virus infection provides protection from apoptosis (J. Taylor and M. Barry unpublished data). Whether the unique N-terminal regions of F1L orthologs provide superior protection against apoptosis or confer a second activity is currently unknown.

All F1L orthologs contain common features that are characteristic of a growing family of proteins known as tail-anchored proteins that includes members of the Bcl-2 family and M11L encoded by myxoma virus(23, 68). Tail-anchored proteins are characterized by having a C-terminal hydrophobic domain flanked by positive charged amino acids that serves as an intracellular localization signal and membrane anchor motif (7, 85). The dual function of the C-terminal region abrogates the need for a N-terminal signal sequence (7, 85). Furthermore, the lack of an N-terminal signal sequence indicates that these proteins are inserted into membranes post-translationally with a defined membrane topology in which the N-terminus is facing the cytoplasm (7, 85). Recently, we characterized the vaccinia virus F1L protein as a new member of the tail-anchored protein family (76). Data from our laboratory indicates that F1L inserts into membranes post-translationally with its N-terminus facing the cytoplasm (76). Furthermore, the C-terminal transmembrane domain of F1L is necessary and sufficient for localization to mitochondria (76, 84). F1L orthologs contain a conserved C-terminal hydrophobic domain flanked by positive charges and a short C-terminal hydrophilic tail (Figure 3-6). Mutation of charged residues in the tail results in F1L localization to the endoplasmic reticulum and loss of its anti-apoptotic function (76). Additional localization studies with F1L orthologs encoded by ectromelia virus, rabbitpox virus, and cowpox virus localize to the mitochondria during virus infection conceivably via the C-terminal domain (76). The fact that F1L, Bcl-2 and M11L are tail-anchored proteins suggests a conserved mechanism for their insertion into the mitochondrial outer membrane.

Following the identification of F1L as a novel inhibitor of apoptosis, we focused our investigation on understanding the anti-apoptotic mechanism of F1L by first attempting to identify F1L-interacting proteins. Since members of the Bcl-2 family are key regulators of the mitochondria, we asked if F1L interacted with Bcl-2 family members. We hypothesized that F1L could function either by inhibiting the function of pro-apoptotic Bcl-2 family proteins or augmenting the inhibitory action of anti-apoptotic Bcl-2 family proteins. Using an immunoaffinity approach, we identified an interaction between Bak and F1L but not Bcl-2, Bcl-XL, Mcl-1 or Bax (Figure 4-4). Additional co-immunoprecipitation experiments confirmed the interaction between F1L and Bak (Figure 4-5). The interaction between F1L and Bak suggested that F1L functioned by modulating Bak activity in order to inhibit apoptosis. Bak is constitutively localized to the outer mitochondrial membrane and under non-apoptotic conditions is held in an inactive state through interactions with Mcl-1 and VDAC2 (11, 46). Following an apoptotic stimulus, Bak is activated either directly or indirectly by BH3-only proteins resulting in exposure of an N-terminal epitope and subsequent homo-oligomerization (30, 31, 86). Since Bak undergoes a multi-step activation process and since both cellular- and viral-encoded anti-apoptotic proteins regulate Bak activation at different points, we sought to determine if F1L inhibited Bak function by addressing the conformational status of Bak (11, 12, 18, 46, 77, 78). Using both a conformationally specific Bak antibody and gel filtration analysis, we found that vaccinia virus infection prevented the N-terminal exposure and oligomerization of Bak in response to staurosporine treatment and this inhibition was dependent on F1L expression (Figure 4-7). Ectopic expression of F1L in the absence of virus infection also prevented the initial N-terminal exposure of Bak following staurosporine treatment indicating that F1L functioned independent of other viral proteins to inhibit Bak activation (Appendix Figure A-6). We also found that F1L modulated Bak activation induced by the BH3-only protein tBid. Mitochondria isolated from vaccinia virus infected MEFs deficient in Bax were protected from Bak homo-oligomerization as monitored by crosslinking with BMH as well as the initial N-terminal exposure of Bak as assessed by limited trypsin proteolysis (Figure 4-8). Collectively, F1L expression prevents Bak activation in response to staurosporine and the BH3-only protein tBid however, the precise mechanism of F1L inhibition of Bak activation is unclear at this time.

5.2 The Anti-apoptotic Mechanism of F1L

Our observations suggest that F1L functions to inhibit apoptosis by regulating the mitochondrial checkpoint thereby inhibiting the release of cytochrome c (Figure 3-9). Similar to F1L, anti-apoptotic Bcl-2 proteins function primarily to inhibit apoptosis by inhibiting the release of cytochrome c (71). Although functioning similar to anti-apoptotic Bcl-2 family members, F1L displays no sequence similarity to Bcl-2 proteins other than the C-terminal mitochondrial targeting motif. The release of cytochrome c is a critical step during apoptosis that occurs as a result of permeabilization of the outer mitochondrial membrane (20, 29, 44, 49). Three mechanisms are proposed to account for mitochondrial outer membrane permeabilization and the release of cytochrome c. One model involves the onset of mitochondrial PT as a result of opening of the PTP that leads to matrix swelling and rupture of the mitochondrial outer membrane (50). A second model proposes the formation of outer membrane pores that allow the passage of cytochrome c (50). The last model involves remodeling of the mitochondrial cristae by a PTP regulated mechanism followed by cytochrome c release that is dependent on the formation of outer membrane pores (70). Although, all three models are distinct, the failure of cells doubly deficient in Bak and Bax to release cytochrome c implicates Bak and Bax in regulating the release of cytochrome c in each model. Inhibition by anti-apoptotic Bcl-2 family members which regulate Bak and Bax activation to inhibit cytochrome c release is also applicable to each model.

In spite of opposing roles, anti- and pro-apoptotic Bcl-2 family proteins share structural similarity to channel forming membrane-spanning bacterial toxins such as colicin and diphtheria toxin (Figure 1-11) (6, 51, 55). This structural similarity has led to the theory that Bcl-2 family members function by forming ion or protein permeable membrane pores. Indeed, Bax, tBid, Bcl-2, and Bcl-XL all display ion-channel activity when incorporated into lipid bilayers or liposomes that requires the two central α-helices (66, 67, 69). The fact that F1L interacts with Bak and inhibits the release of cytochrome c and loss of the inner mitochondrial membrane potential suggests at least a minimal functional conservation between anti-apoptotic Bcl-2 proteins and F1L. The F1L open reading frame however, lacks obvious homology at the primary sequence level to members of the Bcl-2 family including a lack of homology with the BH domains. In contrast, the anti-apoptotic protein M11L encoded by myxoma virus contains a putative BH3 domain and vBcl-2 encoded by fowlpox virus contains obvious BH1 and BH2 domains (2, 82). Since the two central α-helices are proposed to be important for the pore forming function of Bcl-2 family members, we investigated the possible similarity between F1L and Bcl-2 family members at the secondary and tertiary level. Although multiple secondary structure prediction servers including Jpred, PHD, and SAMJ99 predicted that F1L contained multiple helical domains similar to the anti-apoptotic protein Bcl-2, analysis of the F1L sequence with the Superfamily database server provided intriguing insights. The Superfamily database organizes proteins of known structure into multiple classifications using hidden Markov models which is useful for deriving functional and evolutionary insight into a protein of interest (28). The Superfamily database program predicted that F1L contained a membrane translocation domain characteristic of bacterial toxins and Bcl-2 family proteins (13, 47, 55, 58). However, unlike colicin, diphtheria toxin and Bcl-2 proteins whose structure is comprised of two central α-helices surrounded by multiple-helices, the F1L protein is predicted to contain a single α-helix surrounded by seven α-helices similar to *Bacillus thuringiensis* δ-endotoxin (47). The fact that F1L is classified with a similar fold to Bcl-2 family proteins but with only a single central α-helix, suggests that F1L and anti-apoptotic Bcl-2 proteins may function in a similar fashion which is supported by the interaction between F1L and Bak (Figure 4-4, 4-5). A more specific classification with δ-endotoxin and not Bcl-2 family members suggests that F1L may have evolved from a different ancestor than members of the Bcl-2 family or alternatively evolutionary pressure has resulted in significant amino acid changes and structural changes. In contrast, the Superfamily database program predicted the fowlpox vBcl-2 as having structural similarity to cellular Bcl-2 family members while vMIA and M11L failed to be classified into any protein fold superfamily suggesting these proteins have unknown ancestors and therefore may not have retained pore forming function. Overall, this bioinformatics approach has offered some insight not only to the possible mechanism of action of F1L but also suggests Bcl-2 family members, vMIA, M11L and F1L may have been derived from independent ancestors. Only the structural determination of F1L however, will provide solid comparison between the structures of Bcl-2 family members and F1L.

The molecular mechanism of apoptosis regulation by Bcl-2 family members is complex. The essential role of Bak and Bax in the release of cytochrome c indicates that anti-apoptotic Bcl-2 family proteins must counteract Bak and Bax activation (48, 87). There are several well-accepted models for the regulation of Bak and Bax by anti-apoptotic Bcl-2 family members. First, anti-apoptotic Bcl-2 proteins are proposed to directly associate with Bak and Bax preventing Bak and Bax oligomerization and the subsequent release of cytochrome c (3, 11, 63, 86). In this model, BH3-only proteins inactivate anti-apoptotic members of the Bcl-2 family through direct interactions (12, 45). Secondly, anti-apoptotic Bcl-2 proteins are proposed to sequester BH3-only proteins as a direct mechanism to inhibit Bak and Bax activation (9, 12, 45). The prediction that F1L contains a membrane translocation toxin fold similar to Bcl-2 family members and interacts with Bak, suggests that F1L functions in a manner similar to anti-apoptotic Bcl-2 family members.

The interaction between F1L and Bak suggests an obvious potential mechanism by which F1L could inhibit apoptosis. It is plausible that F1L interacts with the inactive version of Bak and inhibits its activation following an apoptotic stimulus. This model is similar to the regulation of Bak by Mcl-1 and VDAC2 (11, 18, 46). Both Mcl-1 and VDAC2 are constitutively associated with the inactive version of Bak. Following an apoptotic stimulus, Bak is activated by liberation from Mcl-1 and VDAC2. Evidence to support a similar mode of action for F1L includes data showing that F1L expression inhibits the initial N-terminal exposure of Bak in response to staurosporine and tBid (Figure 4-7B, 4-8C; Appendix Figure A-6). Furthermore, the constitutive interaction between F1L and Bak lends support to the regulation of Bak by F1L via an inhibitory interaction (Figure 4-4, 4-5). However, the specific nature of the interaction between Bak and F1L is currently unknown. It is conceivable that the interaction between Bak and F1L is direct or may involve other compulsory proteins such as Mcl-1 and VDAC2. The absence of Mcl-1 in our immunoaffinity experiments argue against a role for Mcl-1 in the interaction between F1L and Bak (Figure 4-5). Although speculative at this point, the absence of Mcl-1 in our immunoaffinity assays may reflect the degradation of Mcl-1 which has been shown to occur during UV-light induced apoptosis and infection with adenovirus (18, 54). The functional importance of the interaction between F1L and Bak and whether this interaction is maintained under apoptotic conditions has yet to be determined. Follow-up studies aimed at addressing the interaction between F1L and Bak during apoptosis would be useful since this model predicts that the interaction would be maintained during apoptosis. Furthermore, the identification of amino acid residues or domains within F1L that are critical for the inhibition of apoptosis and interaction with Bak would also be advantageous.

Rather than directly interacting with Bak to modulate its activity, F1L could conceivably target BH3-only proteins. In support of this model, F1L expression prevents the initial exposure of the N-terminal region of Bak, induced by the BH3-only protein tBid (Figure 4-8C). Although this data also supports our first model which proposed that

F1L inhibits Bak activation via an inhibitory interaction with Bak, we can not rule out that F1L may function by sequestering tBid or other BH3-only proteins in order to inhibit Bak and Bax activation. This possible mechanism of F1L-mediated Bak inhibition would be analogous to Bcl-2 which can sequester BH3-only proteins in order to prevent Bak and Bax activation (12, 45). Further experiments focused on identifying an interaction between F1L and tBid would either prove or disprove this model. Additional evidence to support BH3-only protein regulation by F1L stems from data indicating that F1L protects against apoptosis in the presence of Bax. We found that F1L expression clearly inhibited apoptosis in Jurkat cells that express both Bak and Bax and inhibited tBid-induced cytochrome c release from mitochondria purified from vaccinia virus infected MEFs that express both Bak and Bax (Figure 2-3, 2-4, 3-1, 4-7A). Under non-apoptotic conditions, Bax is predominantly cytoplasmic and loosely associated with the mitochondria (89). Following an apoptotic stimulus, BH3-only proteins activate Bax resulting in the exposure of the N-terminal region and the C-terminal transmembrane region of Bax which are necessary for mitochondrial membrane insertion and oligomerization of Bax (3, 8, 89). Following UV-light treatment, vaccinia virus infection but not VV(Cop)ΔF1L infection, prevents the N-terminal exposure of Bax, insertion of Bax into mitochondria and subsequent Bax homo-oligomerization (J. Taylor and M. Barry unpublished results). These data suggest that similar to Bak, the inhibition of Bax activation by F1L occurs upstream of its initial activation step. One model to account for the regulation of Bax is that F1L blocks Bax activation by sequestering BH3-only proteins. Another scenario to account for Bax inhibition is that F1L interacts with Bax however, we have not detected such an interaction (Figure 4-4). It is plausible that F1L and Bax interact following an apoptotic trigger which induces a change in Bax conformation however the fact that F1L prevents the initial N-terminal exposure of Bax induced by UV-light argues against this model although we can not rule out that a conformational change may occur which is not detected by the antibody (J. Taylor and M. Barry unpublished results). An alternative scenario by which F1L inhibits Bax may involve Bak. It is conceivable that F1L regulates Bax activation via its interaction with Bak. In support of this, mitochondria purified from VV(Cop)ΔF1L infected MEFs expressing both Bak and Bax are more sensitive to the effects of tBid when compared to mitochondria purified from VV(Cop)ΔF1L infected MEFs expressing Bak but not Bax (Figure 4-8A). The potential role of Bak in F1L-mediated inhibition of Bax activation could be clarified by performing experiments in MEFs expressing Bax but not Bak and addressing the ability of vaccinia virus, and in particular F1L, to regulate Bax activation.

A model that depicts the anti-apoptotic mechanism of F1L must take into account the possible importance of the PTP in the release of cytochrome c (50). Vaccinia virus strain Copenhagen infection regulates the PTP to prevent loss of the inner mitochondrial membrane potential and release of cytochrome c induced by *t*-butylhydroperoxide and atracyloside, two activators of the PTP (Figure 2-9) (83). Although we have not directly tested the role of F1L in the inhibition of the PTP, it seems reasonable that F1L could play a central role since F1L localizes to mitochondria and functions to maintain mitochondrial integrity during apoptosis. F1L could modulate the PTP by directly interacting with PTP components such as ANT, VDAC, cyclophilin D, or the PBR. The fact that vaccinia virus infection prevents cytochrome c release induced by atracyloside, a direct ligand of the ANT, suggests that vaccinia virus regulates the PTP (Figure 2-9A).

At this time, we have no direct evidence that F1L, or any other vaccinia virus protein, interacts directly with components of the PTP. The interaction between F1L and Bak however, may implicate F1L in PTP regulation. VDAC2, a minor isoform of VDAC, binds to Bak and negatively regulates Bak activity (11). The interaction between VDAC2 and Bak indicates that Bak associates with the PTP and suggests that Bak activity is coupled to the PTP although this has not been tested. Other anti-apoptotic proteins have been identified that interact with pro-apoptotic members of the Bcl-2 family and PTP components. M11L encoded by myxoma virus interacts with the PBR to modulate the PTP and also interacts with Bak to inhibit Bak-induced apoptosis (24, 82). In addition, vMIA encoded by HCMV modulates the PTP by interacting with the ANT and also interacts with Bax to inhibit apoptosis (4, 27, 59). Furthermore, Bcl-XL interacts with VDAC to regulate the PTP and interacts with Bak and Bax to inhibit apoptosis (24, 74, 75). It is therefore possible that F1L interacts with Bak and PTP components in an analogous manner to M11L, vMIA and Bcl-XL. Preliminary experiments assessing the ability of VV(Cop)ΔF1L to inhibit opening of the PTP induced by atracyloside or *t*-butylhydroperoxide will allow us to address the role of F1L in modulating the PTP during virus infection.

To date, viral-encoded mitochondrial inhibitors of apoptosis seem to have a preference for interacting with Bak and Bax rather than BH3-only proteins to regulate apoptosis (36, 53, 79). E1B 19K encoded by adenovirus interacts with Bak and Bax and these interactions are proposed to be the main mechanism by which E1B 19K inhibits apoptosis (17, 56, 77, 78). Binding studies with Kaposi's sarcoma associated virus Bcl-2 indicates that the protein has a much stronger affinity for Bak and Bax BH3 peptides (K_d

<50nM and 980nM respectively) than for BH3 peptide from Bad (K_d 3,900nM) although additional binding experiments with BH3 peptides were not performed to confirm this trend (35, 64). vMIA encoded by HCMV interacts with the conformationally active version Bax yet an interaction between vMIA and tBid has not been detected (4, 59). In addition, a blind proteomic approach identified an interaction between M11L and Bak (82). Our data further supports this model since F1L was observed to specifically interact with Bak but not other anti-apoptotic Bcl-2 family members however, further investigation is essential to address interactions between F1L and BH3-only proteins (Figure 4-4).

The existence of a multi-step process of Bak and Bax activation permits anti-apoptotic viral inhibitors to target a number of steps in the activation process (16). Two well-studied mitochondrial anti-apoptotic viral proteins are E1B 19K encoded by adenovirus and vMIA encoded by HCMV. During adenovirus infection, E1B 19K constitutively interacts with an active version of Bak in which the N-terminus is exposed (17, 18, 77). Following an apoptotic stimulus, E1B 19K retains its interaction with Bak but also extends its inhibition of apoptosis by interacting with Bax in which the N-terminus is exposed (17, 18, 56, 77, 78). In contrast, HCMV encoded vMIA interacts with a conformationally active version of Bax and remarkably vMIA expression activates Bax to form high molecular weight oligomers and yet inhibits Bax-mediated apoptosis (4, 59). An interaction between vMIA and Bak has not been observed and data indicates that vMIA is incapable of inhibiting apoptosis that is dependent on Bak (4). In contrast to E1B 19K and vMIA, F1L constitutively interacts with the inactive version of Bak (Figure 4-4, 4-5). Furthermore, we have not detected an interaction between F1L and Bax. F1L is still capable of inhibiting Bax activation however, indicating that F1L regulates Bax utilizing a different mechanism than E1B 19K and vMIA. Although F1L, E1B 19K and vMIA all function to inhibit apoptosis by regulating the mitochondrial checkpoint, each protein has evolved unique strategies that target specific Bak and Bax activation events. The identification of F1L as a novel inhibitor of apoptosis has served to contribute to our understanding of viral anti-apoptotic strategies and has provided another mechanism by which viruses inhibit apoptosis. Understanding the functional relationship between both F1L and Bak and Bax will undoubtedly provide important insight into understanding the molecular nature of the events that occur at the mitochondria during apoptosis.

5.3 Vaccinia Virus Induces Apoptosis

Members of the poxvirus family encode a broad range of proteins that interfere with apoptosis (Table 1-2). Vaccinia virus encodes multiple proteins that function to regulate distinct steps in the apoptotic pathway. Vaccinia virus encodes both CrmA/Spi-2 which inhibits the activity of caspase 8 and F1L which inhibits the release of cytochrome c from mitochondria during apoptosis (21, 42, 84). Only a few strains of vaccinia virus encode TNFR homologs that sequester TNFα as a mechanism to inhibit the activation of TNFR cellular pathways (61). The fact that vaccinia virus encodes multiple mechanisms to inhibit apoptosis suggests that regulation of this pathway is critical for the virus. The potential requirement for multiple inhibitors of apoptosis may also reflect host-range restrictions in certain cell types that are overcome by expression of specific anti-apoptotic viral proteins. In addition, viral anti-apoptotic proteins may serve dual functions that are implemented in specific cell types or at specific times during the viral infection. For example, the activation of TNFR not only induces apoptosis by way of caspase activation but also triggers an inflammatory response through the activation of NF-κB (52). Thus, it is conceivable that the function of the vaccinia virus TNFR homolog is to prevent both activation of the inflammatory response as well as the inhibition of apoptosis. It is also plausible that vaccinia virus requires multiple specialized inhibitors in order to inhibit apoptosis induced by different stimuli. For example, CrmA/Spi-2 blocks caspase 8 activity to inhibit death receptor-mediated apoptosis while F1L is specialized to inhibit multiple apoptotic cascades including the intrinsic and death receptor apoptotic cascades (21, 42, 76, 84).

During our studies, we observed that infection of Jurkat cells with VV811 and VV(Cop)ΔF1L, both of which are missing F1L, induced apoptosis (Figure 3-10, 4-3 and Appendix Figure A-3, A-5). Currently, there are only a few reports documenting the induction of apoptosis by vaccinia virus (5, 22, 37, 60). These cases typically involve an abortive virus replication cycle in which only early genes are expressed and are isolated to specific cell lines (5, 22, 37, 60). For example, binding of vaccinia virus to the cell surface of Chinese hamster ovary cells is sufficient to induce apoptosis without the need for early gene expression (60). Expression of early proteins during vaccinia virus infection of the murine macrophage cell line J774-G8, the immature B lymphocyte cell line WEHI-231 and immature dendritic cells is sufficient to induce apoptosis (5, 22, 37). Vaccinia virus mutants have also been observed to induce apoptosis. To counter the lethal effects of dsRNA, vaccinia virus encodes E3L, a protein that binds to dsRNA and PKR to inhibit the cellular anti-viral response (39, 73). Data generated with the E3L knockout virus indicates that the production of dsRNA during virus infection induces apoptosis therefore suggesting F1L is unable to inhibit this response (43). Our studies indicate that VV811 or VV(Cop)ΔF1L induced an apoptotic response that proceeded through the mitochondria without prior caspase activation and could be blocked by overexpression of Bcl-2.

The precise mechanism of vaccinia virus triggered apoptosis is unknown. One scenario to account for vaccinia virus induced apoptosis is that the virus encodes a pro-apoptotic protein. Viruses such as human immune deficiency virus, influenza virus and adenovirus encode proteins that induce apoptosis (10, 18, 60, 62, 72, 88). One of the best characterized viral-encoded pro-apoptotic viral proteins is the multifunctional E1A oncoprotein encoded by adenovirus. During adenovirus infection, E1A induces apoptosis which is primarily attributed to induction of a DNA damage response that triggers the degradation of Mcl-1 resulting in Bak activation (18). At this time, we have no evidence to suggest vaccinia virus encodes a pro-apoptotic protein. Based on the fact that VV811 which is missing 55 open reading frames induces apoptosis, it is conceivable that one of the remaining genes is pro-apoptotic.

Alternatively, vaccinia virus infection may activate a currently undefined innate cellular immune response to trigger apoptosis. Our data indicate that VV811 and VV(Cop)ΔF1L infection triggers apoptosis by an unknown mechanism. Several potential viral induced apoptotic triggers are plausible. A post-attachment event is proposed to trigger apoptosis of CHO cells in response to vaccinia virus treatment (60). Early viral protein expression has been documented to induce apoptosis of macrophages, B lymphocytes and immature dendritic cells (5, 22, 37). The accumulation of dsRNA during infection with an E3 deleted vaccinia virus triggers apoptosis (15, 43). With the exception of the E3L knockout virus studies, mitochondria have been identified as a critical regulator of apoptosis induced by vaccinia virus infection (5, 22, 37). Vaccinia virus-induced apoptosis of macrophages is inhibited by infection with a recombinant vaccinia virus expressing Bcl-2 (5). In addition, virus-induced apoptosis of B lymphocytes (WEHI-231) is rescued by infection of cells that stably overexpress Bcl-2 (5). Furthermore, vaccinia virus-induced apoptosis of Chinese hamster ovary cells is delayed by stable expression of E1B 19K or following infection with a recombinant vaccinia virus expressing Bcl-2 (14, 38). Additionally, the induction of apoptosis during VV811 and VV(Cop)ΔF1L can be inhibited by infecting cell lines that stably overexpress Bcl-2 (Figure 4-3, Appendix Figure A-4).

Several models for the activation of the mitochondrial dependent apoptotic cascade as a result of vaccinia virus infection are plausible. It is conceivable that vaccinia virus infection could trigger the activation of BH3-only proteins which transduce signals to the mitochondria during apoptosis (34). The fact that the mitochondrial apoptotic checkpoint is activated during VV811 and VV(Cop)ΔF1L infection without prior caspase activation supports a role for BH3-only proteins other than Bid in the apoptotic pathway induced by vaccinia virus infection (Figure A-2, Appendix Figure A-4). In addition, recent findings in our laboratory indicate that the BH3-only protein Bim is phosphorylated following wildtype vaccinia virus infection clearly demonstrating that infection can activate BH3-only proteins (J. Taylor and M. Barry unpublished results). It is also conceivable that vaccinia virus infection results in the downregulation of anti-apoptotic Bcl-2 proteins to induce apoptosis. In support of this, infection of macrophages and B lymphocytes results in the downregulation of Bcl-XL and Bcl-2 respectively(5, 14, 37). Whether Bcl-XL and Bcl-2 downregulation is a specific viral response during infection or a byproduct of the global shutdown of cellular protein synthesis induced during virus infection is unclear. The possibility that vaccinia virus specifically downregulates anti-apoptotic Bcl-2 family members is appealing since adenovirus infection specifically triggers the downregulation of Mcl-1 to activate apoptosis and relies on E1B 19K overcome Mcl-1 loss (18). It is conceivable that vaccinia virus-induced apoptosis may involve the degradation of Mcl-1 however, steady state levels of Mcl-1 are unaffected during vaccinia virus infection (S. Campbell and M. Barry unpublished results).

The ability of wildtype vaccinia virus infection to induce apoptosis suggests that under some conditions, F1L is incapable of inhibiting apoptosis. A few explanations to account for the inability of F1L to inhibit apoptosis are possible. Since apoptosis induced by wildtype vaccinia virus correlates with an abortive replication, it is possible that F1L might not be expressed (22, 37, 60). While the expression profile of F1L is not clear at this time, we postulate that F1L is an early protein since the genomic sequence upstream of the F1L open reading frame contains sequence elements found in vaccinia virus early promoters and since the anti-apoptotic function ascribed to F1L occurs in the presence of cytosine arabinoside (araC), an inhibitor of vaccinia virus DNA replication and late gene expression (Figure 2-8, panel i and j). On the other hand, if F1L is expressed during the abortive replication cycle, this suggests that F1L is incapable of inhibiting virus-induced apoptosis in these cells. This is an intriguing concept given the fact that expression of E1B 19K and Bcl-2 inhibit apoptosis induced by virus infection in non-permissive cell lines (5, 14, 37, 38). These data may simply reflect the additive effects of the expression of F1L and another mitochondrial inhibitor of apoptosis or that F1L doesn't function in these murine or hamster cells. Alternatively, we can not rule out the possibility that F1L is not functionally redundant to E1B 19K and Bcl-2 which retain the ability to inhibit apoptosis. Indeed, our data currently suggests that F1L interacts with inactive Bak but not Bax whereas both E1B 19K and Bcl-2 interact with the activated version of Bak as well as Bax (11, 17, 18, 33, 56, 63, 77, 78).

5.4 Future Directions

Work that has been undertaken and outlined in this thesis has identified and characterized a new mitochondrial localized inhibitor of apoptosis. A significant step has been made toward understanding the mechanism of action of F1L although a clearer understanding is achievable. Significant questions remain actively pursued in the laboratory with special emphasis on the mechanism by which F1L inhibits apoptosis. In particular, the possible regulation of BH3-only proteins mediated by F1L, the ability of F1L to inhibit Bax mediated apoptosis, and further characterization of the mechanism by which F1L inhibits Bak activation are current topics of research. In addition, the role of F1L in the inhibition of the PTP and the mechanism by which vaccinia virus induces apoptosis with special emphasis on the role of Mcl-1 and Bim are being pursued.

Regrettably, the research covered in this thesis has yet to establish the role of F1L in vaccinia virus virulence. Investigations into the role of vaccinia virus immunomodulatory proteins in virus virulence typically employs a murine model and knockout viruses (40, 81). Accordingly, the contribution of F1L to virus virulence could be achieved using the murine model and comparing virulence of wildtype vaccinia virus versus the virulence of vaccinia virus lacking F1L. Ideally, we would like to employ the ectromelia mouse model which takes advantage of a natural host in which naturally acquired infections are experimentally reproduced (25). Since the ectromelia F1L protein localizes to mitochondria and the inhibition of apoptosis can be ascribed to the last 226 amino acids of the protein, we predict we can utilize the ectromelia mouse model. Collectively, our hope is that dissecting the molecular basis of F1L and its role in viral virulence will lead to an improved understanding of cellular apoptosis and provide insight into the importance of F1L during virus infection.

5.5 References

1. **Adams, J. M., and S. Cory.** 1998. The Bcl-2 protein family: arbiters of cell survival. Science **281**:1322-6.
2. **Afonso, C. L., E. R. Tulman, Z. Lu, L. Zsak, G. F. Kutish, and D. L. Rock.** 2000. The genome of fowlpox virus. J Virol **74**:3815-31.
3. **Antonsson, B., S. Montessuit, B. Sanchez, and J. C. Martinou.** 2001. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem **276**:11615-23.
4. **Arnoult, D., L. M. Bartle, A. Skaletskaya, D. Poncet, N. Zamzami, P. U. Park, J. Sharpe, R. J. Youle, and V. S. Goldmacher.** 2004. Cytomegalovirus cell death suppressor vMIA blocks Bax- but not Bak-mediated apoptosis by binding and sequestering Bax at mitochondria. Proc Natl Acad Sci U S A **101**:7988-93.
5. **Baixeras, E., A. Cebrian, J. P. Albar, J. Salas, A. C. Martinez, E. Vinuela, and Y. Revilla.** 1998. Vaccinia virus-induced apoptosis in immature B lymphocytes: role of cellular Bcl-2. Virus Res **58**:107-13.
6. **Bennett, M. J., and D. Eisenberg.** 1994. Refined structure of monomeric diphtheria toxin at 2.3 A resolution. Protein Sci **3**:1464-75.
7. **Borgese, N., S. Colombo, and E. Pedrazzini.** 2003. The tale of tail-anchored proteins: coming from the cytosol and looking for a membrane. J Cell Biol **161**:1013-9.
8. **Cartron, P. F., H. Arokium, L. Oliver, K. Meflah, S. Manon, and F. M. Vallette.** 2005. Distinct domains control the addressing and the insertion of Bax into mitochondria. J Biol Chem **280**:10587-98.
9. **Chen, L., S. N. Willis, A. Wei, B. J. Smith, J. I. Fletcher, M. G. Hinds, P. M. Colman, C. L. Day, J. M. Adams, and D. C. Huang.** 2005. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell **17**:393-403.
10. **Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J. R. Bennink, and J. W. Yewdell.** 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med **7**:1306-12.
11. **Cheng, E. H., T. V. Sheiko, J. K. Fisher, W. J. Craigen, and S. J. Korsmeyer.** 2003. VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science **301**:513-7.
12. **Cheng, E. H., M. C. Wei, S. Weiler, R. A. Flavell, T. W. Mak, T. Lindsten, and S. J. Korsmeyer.** 2001. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell **8**:705-11.
13. **Choe, S., M. J. Bennett, G. Fujii, P. M. Curmi, K. A. Kantardjieff, R. J. Collier, and D. Eisenberg.** 1992. The crystal structure of diphtheria toxin. Nature **357**:216-22.
14. **Chung, C. S., I. A. Vasilevskaya, S. C. Wang, C. H. Bair, and W. Chang.** 1997. Apoptosis and host restriction of vaccinia virus in RK13 cells. Virus Res **52**:121-32.
15. **Colby, C., C. Jurale, and J. R. Kates.** 1971. Mechanism of synthesis of vaccinia virus double-stranded ribonucleic acid in vivo and in vitro. J Virol **7**:71-6.
16. **Cory, S., D. C. Huang, and J. M. Adams.** 2003. The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene **22**:8590-607.
17. **Cuconati, A., K. Degenhardt, R. Sundararajan, A. Anschel, and E. White.** 2002. Bak and Bax function to limit adenovirus replication through apoptosis induction. J Virol **76**:4547-58.
18. **Cuconati, A., C. Mukherjee, D. Perez, and E. White.** 2003. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. Genes Dev **17**:2922-32.
19. **Cuconati, A., and E. White.** 2002. Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. Genes Dev **16**:2465-78.
20. **Desagher, S., and J. C. Martinou.** 2000. Mitochondria as the central control point of apoptosis. Trends Cell Biol **10**:369-77.
21. **Dobbelstein, M., and T. Shenk.** 1996. Protection against apoptosis by the vaccinia virus SPI-2 (B13R) gene product. J Virol **70**:6479-85.

22. **Engelmayer, J., M. Larsson, M. Subklewe, A. Chahroudi, W. I. Cox, R. M. Steinman, and N. Bhardwaj.** 1999. Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. J Immunol **163:**6762-8.
23. **Everett, H., M. Barry, S. F. Lee, X. Sun, K. Graham, J. Stone, R. C. Bleackley, and G. McFadden.** 2000. M11L: a novel mitochondria-localized protein of myxoma virus that blocks apoptosis of infected leukocytes. J Exp Med **191:**1487-98.
24. **Everett, H., M. Barry, X. Sun, S. F. Lee, C. Frantz, L. G. Berthiaume, G. McFadden, and R. C. Bleackley.** 2002. The Myxoma Poxvirus Protein, M11L, Prevents Apoptosis by Direct Interaction with the Mitochondrial Permeability Transition Pore. J Exp Med **196:**1127-1140.
25. **Fenner, F., and R. Buller.** 1997. Mousepox, p. 535-553, Viral Pathogenesis. Lippincott-Raven Publishers, Philadelphia.
26. **Goebel, S. J., G. P. Johnson, M. E. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete DNA sequence of vaccinia virus. Virology **179:**247-66, 517-63.
27. **Goldmacher, V. S., L. M. Bartle, A. Skaletskaya, C. A. Dionne, N. L. Kedersha, C. A. Vater, J. W. Han, R. J. Lutz, S. Watanabe, E. D. Cahir McFarland, E. D. Kieff, E. S. Mocarski, and T. Chittenden.** 1999. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc Natl Acad Sci U S A **96:**12536-41.
28. **Gough, J., K. Karplus, R. Hughey, and C. Chothia.** 2001. Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. J Mol Biol **313:**903-19.
29. **Green, D. R., and J. C. Reed.** 1998. Mitochondria and apoptosis. Science **281:**1309-12.
30. **Griffiths, G. J., B. M. Corfe, P. Savory, S. Leech, M. D. Esposti, J. A. Hickman, and C. Dive.** 2001. Cellular damage signals promote sequential changes at the N-terminus and BH-1 domain of the pro-apoptotic protein Bak. Oncogene **20:**7668-76.
31. **Griffiths, G. J., L. Dubrez, C. P. Morgan, N. A. Jones, J. Whitehouse, B. M. Corfe, C. Dive, and J. A. Hickman.** 1999. Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. J Cell Biol **144:**903-14.
32. **Hardwick, J. M., and D. S. Bellows.** 2003. Viral versus cellular BCL-2 proteins. Cell Death Differ **10 Suppl 1:**S68-76.
33. **Hsu, Y. T., and R. J. Youle.** 1997. Nonionic detergents induce dimerization among members of the Bcl-2 family. J Biol Chem **272:**13829-34.
34. **Huang, D. C., and A. Strasser.** 2000. BH3-Only proteins-essential initiators of apoptotic cell death. Cell **103:**839-42.
35. **Huang, Q., A. M. Petros, H. W. Virgin, S. W. Fesik, and E. T. Olejniczak.** 2002. Solution structure of a Bcl-2 homolog from Kaposi sarcoma virus. Proc Natl Acad Sci U S A **99:**3428-33.
36. **Huang, Q., A. M. Petros, H. W. Virgin, S. W. Fesik, and E. T. Olejniczak.** 2003. Solution structure of the BHRF1 protein from Epstein-Barr virus, a homolog of human Bcl-2. J Mol Biol **332:**1123-30.
37. **Humlova, Z., M. Vokurka, M. Esteban, and Z. Melkova.** 2002. Vaccinia virus induces apoptosis of infected macrophages. J Gen Virol **83:**2821-32.
38. **Ink, B. S., C. S. Gilbert, and G. I. Evan.** 1995. Delay of vaccinia virus-induced apoptosis in nonpermissive Chinese hamster ovary cells by the cowpox virus CHOhr and adenovirus E1B 19K genes. J Virol **69:**661-8.
39. **Jacobs, B. L., and J. O. Langland.** 1996. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology **219:**339-49.
40. **Johnston, J. B., and G. McFadden.** 2004. Technical knockout: understanding poxvirus pathogenesis by selectively deleting viral immunomodulatory genes. Cell Microbiol **6:**695-705.
41. **Kerr, J. F., A. H. Wyllie, and A. R. Currie.** 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer **26:**239-57.
42. **Kettle, S., A. Alcami, A. Khanna, R. Ehret, C. Jassoy, and G. L. Smith.** 1997. Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1beta-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1beta-induced fever. J Gen Virol **78 (Pt 3):**677-85.
43. **Kibler, K. V., T. Shors, K. B. Perkins, C. C. Zeman, M. P. Banaszak, J. Biesterfeldt, J. O. Langland, and B. L. Jacobs.** 1997. Double-stranded RNA is a trigger for apoptosis in vaccinia virus-infected cells. J Virol **71:**1992-2003.

44. **Kroemer, G., and J. C. Reed.** 2000. Mitochondrial control of cell death. Nat Med **6:**513-9.
45. **Letai, A., M. C. Bassik, L. D. Walensky, M. D. Sorcinelli, S. Weiler, and S. J. Korsmeyer.** 2002. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell **2:**183-92.
46. **Leu, J. I., P. Dumont, M. Hafey, M. E. Murphy, and D. L. George.** 2004. Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol **6:**443-50.
47. **Li, J. D., J. Carroll, and D. J. Ellar.** 1991. Crystal structure of insecticidal delta-endotoxin from Bacillus thuringiensis at 2.5 A resolution. Nature **353:**815-21.
48. **Lindsten, T., A. J. Ross, A. King, W. X. Zong, J. C. Rathmell, H. A. Shiels, E. Ulrich, K. G. Waymire, P. Mahar, K. Frauwirth, Y. Chen, M. Wei, V. M. Eng, D. M. Adelman, M. C. Simon, A. Ma, J. A. Golden, G. Evan, S. J. Korsmeyer, G. R. MacGregor, and C. B. Thompson.** 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell **6:**1389-99.
49. **Liu, X., C. N. Kim, J. Yang, R. Jemmerson, and X. Wang.** 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86:**147-57.
50. **Martinou, J. C., and D. R. Green.** 2001. Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol **2:**63-7.
51. **Muchmore, S. W., M. Sattler, H. Liang, R. P. Meadows, J. E. Harlan, H. S. Yoon, D. Nettesheim, B. S. Chang, C. B. Thompson, S. L. Wong, S. L. Ng, and S. W. Fesik.** 1996. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature **381:**335-41.
52. **Muppidi, J. R., J. Tschopp, and R. M. Siegel.** 2004. Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. Immunity **21:**461-5.
53. **Nava, V. E., E. H. Cheng, M. Veliuona, S. Zou, R. J. Clem, M. L. Mayer, and J. M. Hardwick.** 1997. Herpesvirus saimiri encodes a functional homolog of the human bcl-2 oncogene. J Virol **71:**4118-22.
54. **Nijhawan, D., M. Fang, E. Traer, Q. Zhong, W. Gao, F. Du, and X. Wang.** 2003. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev **17:**1475-86.
55. **Parker, M. W., J. P. Postma, F. Pattus, A. D. Tucker, and D. Tsernoglou.** 1992. Refined structure of the pore-forming domain of colicin A at 2.4 A resolution. J Mol Biol **224:**639-57.
56. **Perez, D., and E. White.** 2000. TNF-alpha signals apoptosis through a bid-dependent conformational change in Bax that is inhibited by E1B 19K. Mol Cell **6:**53-63.
57. **Perkus, M. E., S. J. Goebel, S. W. Davis, G. P. Johnson, E. K. Norton, and E. Paoletti.** 1991. Deletion of 55 open reading frames from the termini of vaccinia virus. Virology **180:**406-10.
58. **Petros, A. M., E. T. Olejniczak, and S. W. Fesik.** 2004. Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta **1644:**83-94.
59. **Poncet, D., N. Larochette, A. L. Pauleau, P. Boya, A. A. Jalil, P. F. Cartron, F. Vallette, C. Schnebelen, L. M. Bartle, A. Skaletskaya, D. Boutolleau, J. C. Martinou, V. S. Goldmacher, G. Kroemer, and N. Zamzami.** 2004. An anti-apoptotic viral protein that recruits Bax to mitochondria. J Biol Chem **279:**22605-14.
60. **Ramsey-Ewing, A., and B. Moss.** 1998. Apoptosis induced by a postbinding step of vaccinia virus entry into Chinese hamster ovary cells. Virology **242:**138-49.
61. **Reading, P. C., A. Khanna, and G. L. Smith.** 2002. Vaccinia virus CrmE encodes a soluble and cell surface tumor necrosis factor receptor that contributes to virus virulence. Virology **292:**285-98.
62. **Roulston, A., R. C. Marcellus, and P. E. Branton.** 1999. Viruses and apoptosis. Annu Rev Microbiol **53:**577-628.
63. **Ruffolo, S. C., and G. C. Shore.** 2003. BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization. J Biol Chem **278:**25039-45.
64. **Sattler, M., H. Liang, D. Nettesheim, R. P. Meadows, J. E. Harlan, M. Eberstadt, H. S. Yoon, S. B. Shuker, B. S. Chang, A. J. Minn, C. B. Thompson, and S. W. Fesik.** 1997. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science **275:**983-6.
65. **Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K. J. Tomaselli, K. M. Debatin, P. H. Krammer, and M. E. Peter.** 1998. Two CD95 (APO-1/Fas) signaling pathways. Embo J **17:**1675-87.

66. **Schendel, S. L., M. Montal, and J. C. Reed.** 1998. Bcl-2 family proteins as ion-channels. Cell Death Differ **5:**372-80.

67. **Schendel, S. L., Z. Xie, M. O. Montal, S. Matsuyama, M. Montal, and J. C. Reed.** 1997. Channel formation by antiapoptotic protein Bcl-2. Proc Natl Acad Sci U S A **94:**5113-8.

68. **Schinzel, A., T. Kaufmann, and C. Borner.** 2004. Bcl-2 family members: intracellular targeting, membrane-insertion, and changes in subcellular localization. Biochim Biophys Acta **1644:**95-105.

69. **Schlesinger, P. H., A. Gross, X. M. Yin, K. Yamamoto, M. Saito, G. Waksman, and S. J. Korsmeyer.** 1997. Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc Natl Acad Sci U S A **94:**11357-62.

70. **Scorrano, L., M. Ashiya, K. Buttle, S. Weiler, S. A. Oakes, C. A. Mannella, and S. J. Korsmeyer.** 2002. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell **2:**55-67.

71. **Scorrano, L., and S. J. Korsmeyer.** 2003. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun **304:**437-44.

72. **Selliah, N., and T. H. Finkel.** 2001. Biochemical mechanisms of HIV induced T cell apoptosis. Cell Death Differ **8:**127-36.

73. **Sharp, T. V., F. Moonan, A. Romashko, B. Joshi, G. N. Barber, and R. Jagus.** 1998. The vaccinia virus E3L gene product interacts with both the regulatory and the substrate binding regions of PKR: implications for PKR autoregulation. Virology **250:**302-15.

74. **Shimizu, S., M. Narita, and Y. Tsujimoto.** 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature **399:**483-7.

75. **Shimizu, S., Y. Shinohara, and Y. Tsujimoto.** 2000. Bax and Bcl-xL independently regulate apoptotic changes of yeast mitochondria that require VDAC but not adenine nucleotide translocator. Oncogene **19:**4309-18.

76. **Stewart, T. L., S. T. Wasilenko, and M. Barry.** 2005. Vaccinia virus F1L protein is a tail-anchored protein that functions at the mitochondria to inhibit apoptosis. J Virol **79:**1084-98.

77. **Sundararajan, R., A. Cuconati, D. Nelson, and E. White.** 2001. Tumor necrosis factor-alpha induces Bax-Bak interaction and apoptosis, which is inhibited by adenovirus E1B 19K. J Biol Chem **276:**45120-7.

78. **Sundararajan, R., and E. White.** 2001. E1B 19K blocks Bax oligomerization and tumor necrosis factor alpha-mediated apoptosis. J Virol **75:**7506-16.

79. **Theodorakis, P., C. D'Sa-Eipper, T. Subramanian, and G. Chinnadurai.** 1996. Unmasking of a proliferation-restraining activity of the anti-apoptosis protein EBV BHRF1. Oncogene **12:**1707-13.

80. **Tulman, E. R., C. L. Afonso, Z. Lu, L. Zsak, G. F. Kutish, and D. L. Rock.** 2004. The genome of canarypox virus. J Virol **78:**353-66.

81. **Turner, P. C., and R. W. Moyer.** 2002. Poxvirus immune modulators: functional insights from animal models. Virus Res **88:**35-53.

82. **Wang, G., J. W. Barrett, S. H. Nazarian, H. Everett, X. Gao, C. Bleackley, K. Colwill, M. F. Moran, and G. McFadden.** 2004. Myxoma virus M11L prevents apoptosis through constitutive interaction with Bak. J Virol **78:**7097-111.

83. **Wasilenko, S. T., A. F. Meyers, K. Vander Helm, and M. Barry.** 2001. Vaccinia virus infection disarms the mitochondrion-mediated pathway of the apoptotic cascade by modulating the permeability transition pore. J Virol **75:**11437-48.

84. **Wasilenko, S. T., T. L. Stewart, A. F. Meyers, and M. Barry.** 2003. Vaccinia virus encodes a previously uncharacterized mitochondrial-associated inhibitor of apoptosis. Proc Natl Acad Sci U S A **100:**14345-50.

85. **Wattenberg, B., and T. Lithgow.** 2001. Targeting of C-terminal (tail)-anchored proteins: understanding how cytoplasmic activities are anchored to intracellular membranes. Traffic **2:**66-71.

86. **Wei, M. C., T. Lindsten, V. K. Mootha, S. Weiler, A. Gross, M. Ashiya, C. B. Thompson, and S. J. Korsmeyer.** 2000. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev **14:**2060-71.

87. **Wei, M. C., W. X. Zong, E. H. Cheng, T. Lindsten, V. Panoutsakopoulou, A. J. Ross, K. A. Roth, G. R. MacGregor, C. B. Thompson, and S. J. Korsmeyer.** 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science **292:**727-30.

88. **White, E.** 2001. Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus. Oncogene **20:**7836-46.

89. **Wolter, K. G., Y. T. Hsu, C. L. Smith, A. Nechushtan, X. G. Xi, and R. J. Youle.** 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol **139:**1281-92.

Appendix – Supporting Methods and Results

A-1 Stable Expression of F1L Inhibits Staurosporine-induced Loss of the Inner Mitochondrial Membrane Potential.

BMGneo Clone	% TMRE Loss	HA-F1L Clone	%TMRE Loss
3B2*	40%	1B6	24%
3D3*	40%	1C5	57%
3D6	38%	1E2	8%
3D10	40%	1E10	8%
4B11	52%	1G4*	6%
4C3	47%	2F3	19%
4C7	43%	2F6	20%
4C8	28%	2F10*	5%
4C9	39%	2F11	10%
4C11	6%	1G5	22%
Jurkat Parental Cells	44%		

Table A-1 Stable expression of F1L is sufficient to block the loss of the inner mitochondrial membrane potential.
Jurkat clones transfected with BMGneo or BMGneo-HA-F1L-N were treated with 1μM staurosporine for seventy-five minutes and the loss of the inner mitochondrial membrane potential was monitored by measuring the uptake of TMRE. (*) denotes the stable cell clones used in further experiments.

A.2 Transcription Profile of F1L Stable Clones

A.2.1 Method for RT-PCR.

Total RNA was isolated with Trizol according to the manufacturers protocol (Invitrogen). PolyA RNA was reversed transcribed with Superscript II RNAse H reverse transcriptase (Invitrogen) using the oligo-d_T primer (Invitrogen) according to the manufacturers protocol. The cDNA corresponding to β-Actin was amplified using the forward primer (5'-GCACCACACCTTCTACAATGAG-3') and reverse primer (5'-AAATAGCACAGCCTGGATAGCAAC-3'). F1L transcripts were amplified using a forward primer containing the nucleotide sequence for the HA-epitope appended to the first 15 nucleotides of the vaccinia virus strain Copenhagen F1L gene (5'-GTCGAC ATGTACCCATACGATGTTCCAGATTACGCTCTTATGTTGTCGATGTTTATG-3') and the reverse primer (5'-CTCGAGTTATCCTATCATGTATTT-3'). The PCR products were resolved by agarose gel analysis and visualized with ethidium bromide.

A.2.2 Results.

Jurkat cells were stably transfected with BMGneo or BMGneo-F1L-HA-N and subsequently cloned by serial dilution and expanded. Four G418 insensitive clones from each transfected cell population were chosen to address the presence of F1L transcripts. As shown in Figure A-1, all four BMGneo clones (3B2, 3D3, 3D6, and 3D10) were negative for the presence of F1L transcripts whereas the clones stably transfected with BMGneo-F1L-HA-N (2F10, 1G4, 2F6, 1E10) contained F1L transcripts. As a control, all clones showed similar levels of β-actin indicating equal loading of samples (Figure A-1).

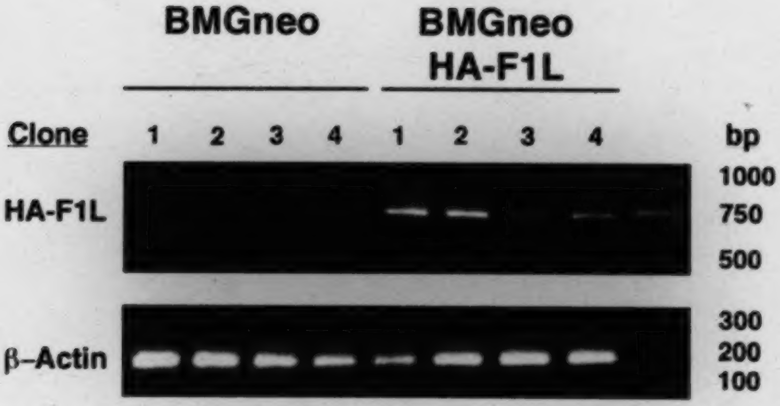

BMGneo
BMGneo HA-F1L
Clone
1
2
3
4
1
2
3
4
bp
HA-F1L
β–Actin
1000
750
500
300
200
100

Figure A-1 Transcript profile of Jurkat cells stably expressing F1L.
Agarose gel analysis of RT-PCR samples from RNA purified from Jurkat clones stably transfected with BMGneo or BMGneo-HA-F1L using primers directed against F1L or β-Actin. BMGneo clones are as follows. Clone 1, 3B2; Clone 2, 3D3; Clone 3, 3D6; Clone 4, 3D10; HA-F1L clones are as follows. Clone 1, 2F10; Clone 2, 1G4; Clone 3, 2F6; Clone 4, 1E10.

A.3 Stable Expression of F1L Protects Cells from Granzyme B-induced Apoptosis

Following the recognition of virus-infected cells, CTL and NK cells release lytic granules that contain death-inducing proteases including granzyme B (11). Granzyme B enters the target cell cytoplasm and triggers the intrinsic apoptotic cascade by cleaving Bid, Mcl-1 and caspase-3 (1, 3, 4, 7, 9, 10). Since we had previously shown that vaccinia virus infection protected against granzyme B cleaved tBid-induced cytochrome c release, we asked if F1L protected cells from granzyme B-induced apoptosis (13).

A.3.1 Method for the Induction of Granzyme B-induced Apoptosis.

Jurkat cells (1×10^6) were treated with purified granzyme B (250ng) and adenovirus (10 PFU/cell) and incubated at 37°C for either 1 or 2 hours (2, 8). The loss of the inner mitochondrial membrane potential was assessed by monitoring TMRE fluorescence and DNA fragmentation was monitored by TUNEL assay as previously described (13).

A.3.2 Results.

To further characterize the ability of F1L to inhibit apoptosis, we asked if ectopic expression of F1L inhibited granzyme B-induced apoptosis. We took advantage of the stable cell lines generated to stably express F1L. We treated the stable cell lines with adenovirus and granzyme B and monitored DNA fragmentation by TUNEL assay. In the absence of adenovirus and granzyme B, all cell lines demonstrated low levels of DNA fragmentation (Figure A-2 panels a, e, g, and i). As controls, cells treated with either

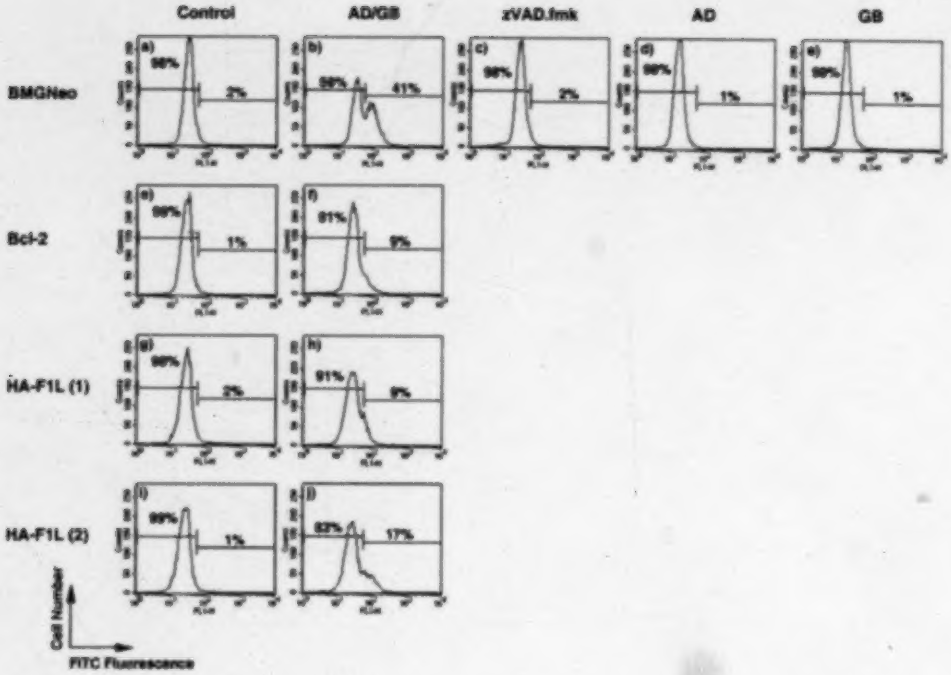


Figure A-2 Stable expression of F1L prevents granzyme B-induced DNA fragmentation.

Jurkat cells were treated with granzyme B (250 ng) and adenovirus (10 PFU/cell) for two hours and DNA fragmentation was assessed by TUNEL assay. (a) Untreated Jurkat cells stably transfected with BMGneo (clone 3D6); (b) Jurkat cells stably transfected with BMGneo (clone 3D6) treated with granzyme B (GB) and adenovirus (AD); (c) Jurkat cells stably transfected with BMGneo treated with GB and AD in the presence of 100μM zVAD.fmk; (d) Jurkat cells stably transfected with BMGneo treated with AD; (e) Jurkat cells stably transfected with BMGneo treated with GB; (f) Jurkat cells overexpressing Bcl-2; (g) Jurkat cells overexpressing Bcl-2 treated with GB and AD; (g) Jurkat cells stably expressing HA-F1L (clone 2F10); (h) Jurkat cells stably expressing HA-F1L (clone 2F10) treated with GB and AD; (i) Jurkat cells stably expressing HA-F1L (clone 1G4); (j) Jurkat cells stably expressing HA-F1L (clone 1G4) treated with GB and AD.

adenovirus or granzyme B alone displayed low levels of DNA fragmentation (Figure A-2 panel d and e). Jurkat cells stably transfected with the empty vector BMGneo and treated with adenovirus and granzyme B showed 41% of the cells positive for DNA fragmentation (Figure A-2 panel b). DNA fragmentation in response to adenovirus and granzyme B treatment was inhibited by the presence of zVAD.fmk, a broad spectrum caspase inhibitor (Figure A-2 panel c). As expected cells overexpressing Bcl-2 were protected from DNA fragmentation in response to adenovirus and granzyme B treatment (Figure A-2 panel f). Importantly, two cell lines stably expressing F1L and treated with adenovirus and granzyme B displayed only 9% and 17% DNA fragmentation (Figure A-2 panel h and i) indicating that F1L expression could inhibit apoptosis induced by granzyme B.

To confirm this finding, we monitored the ability of F1L to inhibit the loss of the inner mitochondrial membrane potential induced by granzyme B by measuring the uptake of TMRE (Figure A-3). All untreated cells displayed high levels of TMRE fluorescence (Figure A-3 panels a, e, g, and i). The treatment of cells stably transfected with the BMGneo vector and treated with adenovirus or granzyme B alone retained high levels of TMRE fluorescence (Figure A-3 panel d and e). As a control, cells stably transfected with BMGneo were treated with ClCCP which resulted in complete loss of the inner mitochondrial membrane potential (Figure A-3 panel c). Treatment of Jurkat cells stably transfected with the BMGneo vector and treated with adenovirus and granzyme B resulted in 33% of the cells showing a decrease in the uptake of TMRE (Figure A-3 panel b). Cells overexpressing Bcl-2 were protected from adenovirus and granzyme B induced loss of the inner mitochondrial membrane potential (Figure A-3 panel f). Treatment of

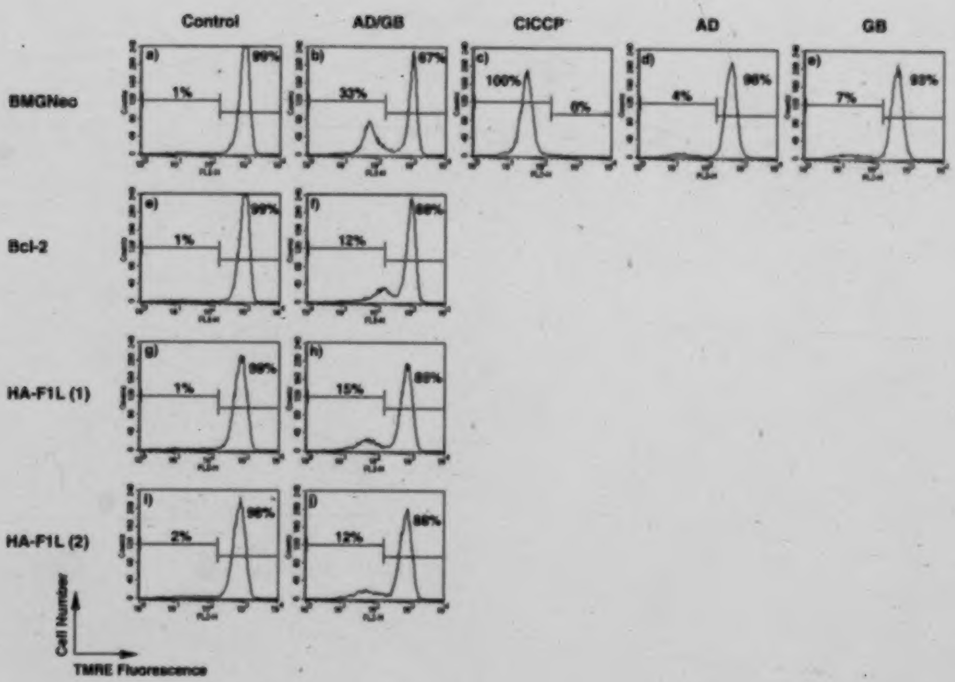


Figure A-3 Stable expression of F1L prevents granzyme B-induced loss of the inner mitochondrial membrane potential.

Jurkat cells were treated with granzyme B (250 ng) and adenovirus (10 PFU/cell) for 1 hour and the loss of the inner mitochondrial membrane potential was monitored by TMRE uptake. (a) Untreated Jurkat cells stably transfected with BMGneo (clone 3D6); (b) Jurkat cells stably transfected with BMGneo (clone 3D6) treated with granzyme B (GB) and adenovirus (AD); (c) Jurkat cells stably transfected with BMGneo (clone 3D6) treated with the membrane uncoupler ClCCP; (d) Jurkat cells stably transfected with BMGneo treated with AD; (e) Jurkat cells stably transfected with BMGneo (clone 3D6) treated with GB; (f) Jurkat cells overexpressing Bcl-2; (g) Jurkat cells overexpressing Bcl-2 treated with GB and AD; (g) Jurkat cells stably expressing HA-F1L (clone 2F10); (h) Jurkat cells stably expressing HA-F1L (clone 2F10) treated with GB and AD; (i) Jurkat cells stably expressing HA-F1L (clone 1G4); (j) Jurkat cells stably expressing HA-F1L (clone 1G4) treated with GB and AD.

two clones stably expressing F1L with adenovirus and granzyme B resulted in only 15% and 12% of the cells undergoing loss of the inner mitochondrial membrane potential (Figure A-3 panel h and i). Taken together, these results indicated that ectopic expression of F1L inhibited apoptosis induced by granzyme B.

A.4 VV811 Induces Apoptosis in Infected Cells Directed Through the Mitochondrial Cascade

Since we had observed that VV811 infection induced apoptosis as measured by quantifying levels of DNA fragmentation (Figure 3-10A), we asked whether VV811 induced other biochemical features of apoptosis (14). We focused on the mitochondrial dependent apoptotic cascade since we had observed that either stable expression of F1L, overexpression of Bcl-2 or loss of Bak and Bax expression could inhibit VV811-induced apoptosis (Figure 3-10B) (14).

A.4.1 Method for the Induction of VV811-induced Apoptosis.

Jurkat cells were infected with VV65, VV811, or VV759 at a MOI or 10 PFU/cell in 200μL RHFM. Following incubation for 1 hour, cells were supplemented with an additional 1.3mL RHFM and incubated for and additional 4, 9, and 14 hours at 37°C. Where indicated, 100 μM zVAD.fmk (Kamiya Biomedical) was added 1 hour following infection.

The loss of the inner mitochondrial membrane potential was monitored by measuring the uptake of TMRE (13). Whole cell lysates and fractionated lysates were analyzed by SDS-Page and PARP cleavage, caspase activation and cytochrome c release were monitored by western blot analysis (13, 14).

A.4.2 Results.

Since we had shown that VV811 induced DNA fragmentation following a fifteen hour infection (Figure 3-10A), we asked if VV811 induced other hallmark features of apoptosis over the same time period. We infected Jurkat cells with VV(Cop), VV759 or VV811 for five, ten, and fifteen hours and monitored post-mitochondrial events including PARP cleavage, caspase 3 activation, caspase 9 cleavage, and the release of SMAC and cytochrome c from the mitochondria. As shown in Figure A-4A, Jurkat cells mock infected showed no PARP, caspase 3 or caspase 9 cleavage and maintained both SMAC and cytochrome c in the mitochondrial fraction (Figure A-4A). As expected, cells infected with VV(Cop) or VV759 were protected from the cleavage of PARP, activation of caspases 3 and 9 and the release of SMAC and cytochrome c (Figure A-4B and C). In the presence of VV811 however, full length PARP was cleaved to its 89 kDa cleaved form (Figure A-4D). Infection of Jurkat cells with VV811 also showed obvious caspase 3 and caspase 9 cleavage and the release of SMAC and cytochrome c from the mitochondria (Figure A-4D). The release of SMAC and cytochrome c occurred in the presence of the broad spectrum caspase inhibitor, zVAD.fmk, indicating that VV811 triggered apoptosis that did not require caspase activation prior to mitochondrial permeabilization.

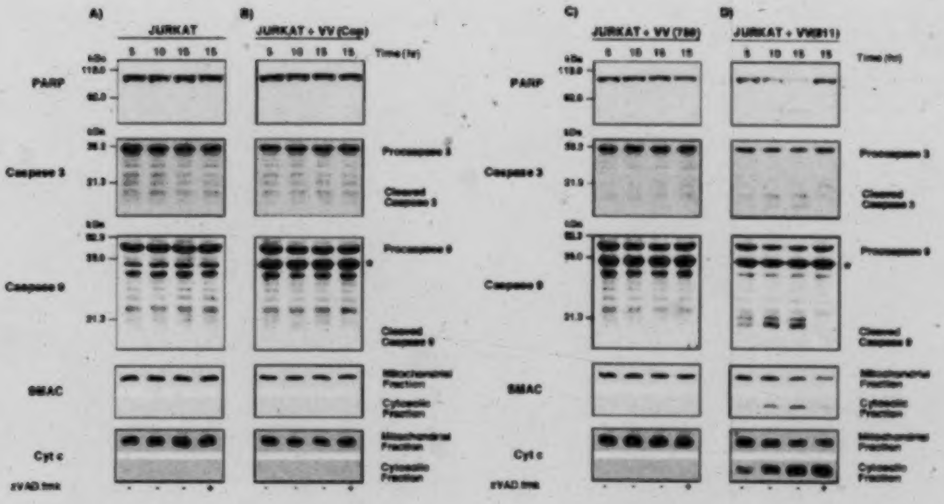

A)
JURKAT
B)
JURKAT + VV(Cop)
C)
JURKAT + VV(759)
D)
JURKAT + VV811)
5 10 15 15
Time (hr)
kDa
PARP
Caspase 3
Procaspase 3
Cleaved Caspase 3
Caspase 9
Procaspase 9
Cleaved Caspase 9
*
SMAC
Mitochondrial Fraction
Cytosolic Fraction
Cyt c
zVAD.fmk
- - - +

Figure A-4 VV811 infection induces the hallmark features of mitochondrial dependent apoptosis.

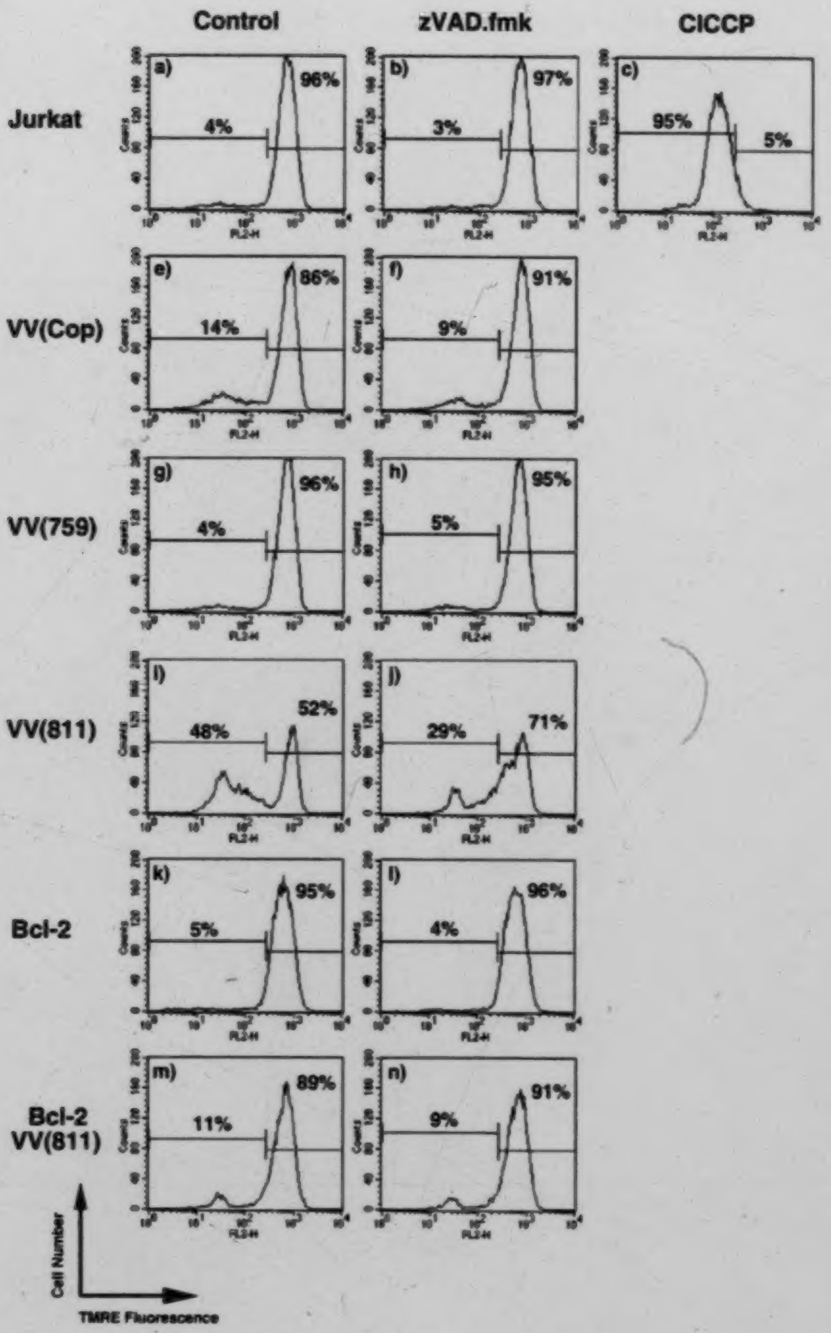
Jurkat cells were either mock infected or infected with VV(Cop), VV759 or VV811 at an MOI of 10 in the presence or absence of zVAD.fmk. At the indicated times, cells lysates were generated and the cleavage of PARP, caspase 3 and 9 were monitored by western blot analysis. (*) denotes a cross reacting protein present in virus-infected lysates. To detect caspase 3 and 9 cleavage and cytochrome c release, cell were fractionated into membranous and cytosolic fractions and the translocation of cytochrome c was assessed by western blot. (A) Jurkat cells mock infected; (B) Jurkat cells infected with VV(Cop); (C) Jurkat cells infected with VV759; (D) Jurkat cells infected with VV811.

The release of SMAC and cytochrome c in response to VV811 infection suggested that mitochondrial integrity is compromised as a result of vaccinia virus infection. To further characterize the ability of VV811 to induce apoptosis, and confirm the involvement of the mitochondria, we monitored the loss of the inner mitochondrial membrane potential in response to virus infection. Jurkat cells were mock infected or infected with VV811 for 15 hours and the loss of the inner mitochondrial membrane potential was monitored by TMRE uptake. As shown in Figure A-5, in the absence of infection, 96% of the cells displayed high levels of TMRE uptake (Figure A-5 panel a). Jurkat cells infected with VV(Cop) or VV759 preserved high levels of TMRE fluorescence (Figure A-5 panel e and g) whereas 48% of Jurkat cells infected with VV811 showed a reduction in the levels of TMRE uptake that could be partially restored by the addition of zVAD.fmk (Figure A-5 panel i and j). As a control, the majority of Jurkat cells treated with ClCCP underwent loss of the inner mitochondrial membrane potential (Figure A-5 panel c). Importantly, infection of Jurkat cells overexpressing Bcl-2 also protected cells against VV811-induced loss of the inner mitochondrial membrane potential confirming previous results that VV811 induced apoptosis was directed through the mitochondria (Figure A-5 panels m and n). Overall these results suggested that VV811 induced apoptosis in infected cells that proceeded through mitochondria.

A.5 F1L Expression Inhibits Staurosporine-induced Bak Activation

In view of our findings that F1L interacted with Bak in the absence of virus infection and that expression of F1L was necessary for vaccinia virus to inhibit the

Figure A-5 VV811 induces the loss of the inner mitochondrial membrane potential. Following a fifteen hour infection, the loss the inner mitochondrial membrane potential was determined by TMRE uptake. (A) Jurkat cells were either mock infected or infected with VV(Cop), VV(759) or VV(811). (a) Uninfected Jurkat cells; (b) Jurkat cells mock infected in the presence of zVAD.fmk; (c) Jurkat cells treated with ClCCP; (d) Jurkat cells infected with VV(Cop); (e) Jurkat cells infected with VV(Cop) in the presence of zVAD.fmk; (f) Jurkat cells infected with VV(759); (g) Jurkat cells infected with VV(759) in the presence of zVAD.fmk; (i) Jurkat cells infected with VV(811); (j) Jurkat cells infected with VV(811) in the presence of zVAD.fmk. (B) Jurkat cells overexpressing Bcl-2 prevent VV(811)-induced loss of the inner mitochondrial membrane potential. (a) Jurkat cells overexpressing Bcl-2; (b) Jurkat cells overexpressing Bcl-2 in the presence of zVAD.fmk; (c) Jurkat cells overexpressing Bcl-2 infected with VV(811); (d) Jurkat cells overexpressing Bcl-2 infected with VV(811) in the presence of zVAD.fmk.


Control
zVAD.fmk
ClCCP
Jurkat
VV(Cop)
VV(759)
VV(811)
Bcl-2
Bcl-2 VV(811)
a)
4%
96%
b)
3%
97%
c)
95%
5%
e)
14%
86%
f)
9%
91%
g)
4%
96%
h)
5%
95%
i)
48%
52%
j)
29%
71%
k)
5%
95%
l)
4%
96%
m)
11%
89%
n)
9%
91%
Counts
FL2-H
Cell Number
TMRE Fluorescence

activation of Bak, we asked if F1L expression in the absence of virus infection could inhibit Bak activation (12).

A.5.1 Method for Monitoring the N-terminal Exposure of Bak.

Jurkat cells were induced to undergo apoptosis by the addition of 0.5μM staurosporine for two hours. The conformational status of Bak was assessed as previously described (5, 6, 12) .

A.5.2 Results.

We utilized Jurkat cells stably expressing a HA-tagged version of F1L (clone 2F10) which were previously shown to inhibit staurosporine-induced cytochrome c release (14). We assessed the activation of Bak using a conformation specific antibody directed against the N-terminus of Bak and monitored the appearance of activated Bak by flow cytometry. In the absence of staurosporine, all cells displayed low levels of background fluorescence associated with non-specific antibody staining (Figure A-6). The addition of staurosporine to BMGneo stable clones triggered the exposure of the N-terminus of Bak (Figure A-6 panel a). Treatment of Jurkat cells devoid of Bak and Bax with staurosporine exhibited similar fluorescent profiles observed in untreated samples (Figure A-6 panel d). As a control, Jurkat cells overexpressing Bcl-2 were protected from staurosporine induced Bak N-terminus exposure (Figure A-6 panel c). Importantly, Jurkat cells stably expressing HA-F1L prevented the N-terminal exposure of Bak following the addition of staurosporine indicating that F1L in the absence of virus infection inhibited the pro-apoptotic function of Bak (Figure A-6 panel b).

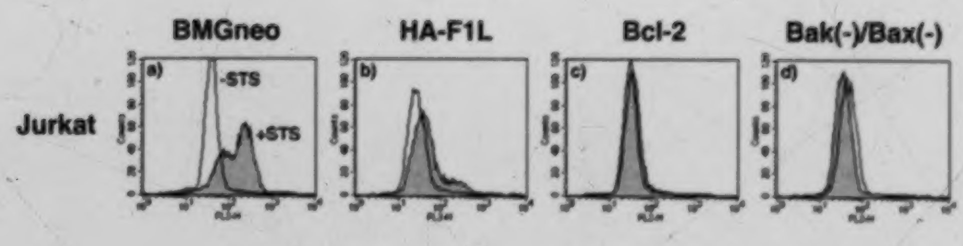


Figure A-6 Stable expression of F1L prevents staurosporine-induced N-terminal exposure of Bak.

Jurkat cells were treated with 0.5μM staurosporine for 2 hours and the N-terminal exposure of Bak was assessed using a conformationally specific anti-Bak antibody. (a) Jurkat cells stably transfected with BMGneo (clone 3D6); (b) Jurkat cells stably expressing HA-F1L (clone 2F10); (c) Jurkat cells overexpressing Bcl-2; (d) Jurkat cells lacking Bak and Bax. Untreated cells (open histogram); staurosporine treated cells (shaded histogram).

A.6 References

1. **Barry, M., J. A. Heibein, M. J. Pinkoski, S. F. Lee, R. W. Moyer, D. R. Green, and R. C. Bleackley.** 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol **20:**3781-94.
2. **Caputo, A., J. C. Parrish, M. N. James, J. C. Powers, and R. C. Bleackley.** 1999. Electrostatic reversal of serine proteinase substrate specificity. Proteins **35:**415-24.
3. **Darmon, A. J., T. J. Ley, D. W. Nicholson, and R. C. Bleackley.** 1996. Cleavage of CPP32 by granzyme B represents a critical role for granzyme B in the induction of target cell DNA fragmentation. J Biol Chem **271:**21709-12.
4. **Darmon, A. J., D. W. Nicholson, and R. C. Bleackley.** 1995. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. Nature **377:**446-8.
5. **Griffiths, G. J., B. M. Corfe, P. Savory, S. Leech, M. D. Esposti, J. A. Hickman, and C. Dive.** 2001. Cellular damage signals promote sequential changes at the N-terminus and BH-1 domain of the pro-apoptotic protein Bak. Oncogene **20:**7668-76.
6. **Griffiths, G. J., L. Dubrez, C. P. Morgan, N. A. Jones, J. Whitehouse, B. M. Corfe, C. Dive, and J. A. Hickman.** 1999. Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. J Cell Biol **144:**903-14.
7. **Han, J., L. A. Goldstein, B. R. Gastman, C. J. Froelich, X. M. Yin, and H. Rabinowich.** 2004. Degradation of Mcl-1 by granzyme B: implications for Bim-mediated mitochondrial apoptotic events. J Biol Chem **279:**22020-9.
8. **Hanna, W. L., X. Zhang, J. Turbov, U. Winkler, D. Hudig, and C. J. Froelich.** 1993. Rapid purification of cationic granule proteases: application to human granzymes. Protein Expr Purif **4:**398-404.
9. **Heibein, J. A., I. S. Goping, M. Barry, M. J. Pinkoski, G. C. Shore, D. R. Green, and R. C. Bleackley.** 2000. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. J Exp Med **192:**1391-402.
10. **Quan, L. T., M. Tewari, K. O'Rourke, V. Dixit, S. J. Snipas, G. G. Poirier, C. Ray, D. J. Pickup, and G. S. Salvesen.** 1996. Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B. Proc Natl Acad Sci U S A **93:**1972-6.
11. **Trapani, J. A., and M. J. Smyth.** 1993. Killing by cytotoxic T cells and natural killer cells: multiple granule serine proteases as initiators of DNA fragmentation. Immunol Cell Biol **71 (Pt 3):**201-8.
12. **Wasilenko, S. T., L. Banadyga, D. Bond, and M. Barry.** 2005. The vaccinia virus F1L protein interacts with the pro-apoptotic protein Bak and inhibits Bak activation. Submitted to Journal of Virology.
13. **Wasilenko, S. T., A. F. Meyers, K. Vander Helm, and M. Barry.** 2001. Vaccinia virus infection disarms the mitochondrion-mediated pathway of the apoptotic cascade by modulating the permeability transition pore. J Virol **75:**11437-48.
14. **Wasilenko, S. T., T. L. Stewart, A. F. Meyers, and M. Barry.** 2003. Vaccinia virus encodes a previously uncharacterized mitochondrial-associated inhibitor of apoptosis. Proc Natl Acad Sci U S A **100:**14345-50.